

**PCT**WORLD INTELLECTUAL PROPERTY  
International Bureau

WO 9608514A1

## INTERNATIONAL APPLICATION PUBLISHED UNDER T1

(51) International Patent Classification 6 : <b>C07K 16/00, 16/30, 16/18, 14/00, A61K 39/395, 41/00, 49/00, 51/00, G01N 33/574, C12N 15/02, C12P 21/08</b>		A1	(11) International Publication Number: <b>WO 96/08514</b> (43) International Publication Date: <b>21 March 1996 (21.03.96)</b>
(21) International Application Number: <b>PCT/US95/11554</b> (22) International Filing Date: <b>12 September 1995 (12.09.95)</b>  (30) Priority Data: <b>08/304,524 12 September 1994 (12.09.94) US</b>		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TI, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71)(72) Applicants and Inventors: <b>TSANG, Kwong, Y. [US/US]; 5420 York Lane, Bethesda, MD 20814 (US). ARLEN, Myron [US/US]; 81 Wensley Drive, Great Neck, NY 11020 (US).</b>  (74) Agent: <b>REINGOLD, Jesse, D.; Rosenman &amp; Colin L.L.P., 575 Madison Avenue, New York, NY 10022 (US).</b>			
(54) Title: <b>MONOCLONAL ANTIBODIES AGAINST HUMAN COLON CARCINOMA-ASSOCIATED ANTIGENS AND USES THEREFOR</b>			
(57) Abstract  Monoclonal antibodies, in particular 33.28 and 31.1, and chimeric antibodies, in particular mouse/human chimeric Chi #1 specific for glycoprotein antigens of colon carcinoma-associated antigens which are immunogenic in humans, are disclosed. Such antibodies, and fragments and derivatives thereof, are useful in immunodiagnosis and immunotherapy of human colon, breast, and ovarian cancer, and for purification of antigens which can serve as immunotherapeutic agents. Methods of detecting the colon carcinoma-associated antigen in a sample, and methods for treating subjects having colon, breast, and ovarian carcinomas are disclosed.			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of patent applications under the PCT.

AT	Austria	GB	United Kingdom
AU	Australia	GE	Georgia
BB	Barbados	GN	Guinea
BE	Belgium	GR	Greece
BF	Burkina Faso	HU	Hungary
BG	Bulgaria	IE	Ireland
BJ	Benin	IT	Italy
BR	Brazil	JP	Japan
BY	Belarus	KE	Kenya
CA	Canada	KG	Kyrgyzstan
CF	Central African Republic	KP	Democratic People's Republic of Korea
CG	Congo	KR	Republic of Korea
CH	Switzerland	KZ	Kazakhstan
CI	Côte d'Ivoire	LI	Liechtenstein
CM	Cameroon	LK	Sri Lanka
CN	China	LU	Luxembourg
CS	Czechoslovakia	LV	Latvia
CZ	Czech Republic	MC	Monaco
DE	Germany	MD	Republic of Moldova
DK	Denmark	MG	Madagascar
ES	Spain	ML	Mali
FI	Finland	MN	Mongolia
FR	France		
GA	Gabon		

**MONOCLONAL ANTIBODIES AGAINST HUMAN COLON  
CARCINOMA-ASSOCIATED ANTIGENS AND USES THEREFOR**

This application is a continuation-in-part of U.S. application Serial No. 08/159,836 filed November 30, 1993, which is a continuation-in-part of U.S. application Serial No. 08/117,430, filed September 7, 1993, now abandoned, which is a continuation-in-part of U.S. application Serial No. 07/670,816, filed March 18, 1991, now abandoned, which is a continuation-in-part of U.S. application Serial No. 07/176,337, filed March 31, 1988, now abandoned.

10

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

This invention, in the field of immunology and medicine, relates to new hybridoma lines and the monoclonal antibodies (mAbs) they secrete which are specific for clinically defined colon carcinoma-associated antigens. The Mabs are useful in vivo for immunodetection and immunotherapy of colon carcinoma as well as for the detection and purification of colon carcinoma-associated antigens.

**Description of the Background Art**

During the process of oncogenesis, a number of cell-surface molecules or markers appear on cells. Such tumor-related markers include oncofetoproteins, neo-glycoproteins, sphingolipids, and modifications of existing surface proteins. Such new or altered structures are often shed from the tumor cell surface and appear in the serum or in other biological fluids. The detection of any of these substances or "tumor markers" serves as the basis for diagnosing or monitoring the progress of neoplastic disease.

20

Early animal studies demonstrated that, among these tumor markers, a subset of tumor membrane protein or glycoprotein antigens were immunogenic. Upon appropriate reintroduction into the tumor-bearing host, typically after surgical removal of the primary tumor, such antigens could effectively block the establishment of new tumor growth.

An attempt to use similarly derived tumor-associated antigens in humans was made by Hollinshead and Stewart using a relatively purified membrane preparation in patients with lung cancer (Stewart, T.H.M. *et al.*, Ann. N.Y. Acad. Sci. 277:436 (1976)). These studies were later expanded to include 5 patients with melanoma and colon carcinoma, wherein different pooled allogeneic tumor preparations were administered in combination with complete Freund's adjuvant (Hollinshead, A.C. *et al.*, Cancer 49:1387 (1982); Hollinshead, A.C. *et al.*, Cancer 56:480 (1985)).

The use of Freund's adjuvant was based on observations that normal 10 tissue antigens with this adjuvant produced severe autoimmune responses in animal recipients, whereas in the absence of this adjuvant, no adverse reactions were seen. The adjuvant was thought to promote antigen processing by host macrophages as well as prolong the stimulatory action of the antigen at the site of its deposition (see, for example, Roitt, I., Essential Immunology, 15 6th Ed., Blackwell Scientific Publications, Oxford (1988)).

The above observations served as the basis for early clinical trials using specific human tumor membrane proteins and glycoproteins as tumor "vaccines." Various of the tumor-associated antigens which had been isolated and characterized could prolong survival and, in some cases, produce regression 20 of metastatic disease.

With the advent of monoclonal antibody (mAb) technology, it has become possible to obtain pure antibody populations which permit better purification and characterization of the various tumor markers and tumor-associated antigens that are useful for immunodiagnosis or immunotherapy. Many 25 mAbs have been described that have varying degrees of selectivity for tumor antigens (versus normal cell surface markers); some of these tumor antigens are broadly represented across several or many tumor types, whereas others appear to be truly tumor-specific. A number of these mAb-tumor antigen systems are described below.

30 Herlyn *et al.*, Proc. Natl. Acad. Sci. USA 76:1438 (1979), discloses two mAbs obtained by immunizing mice with human colorectal carcinoma (CRC) cells. The mAbs have selective reactivity with human CRC cells. One mAb, 1083-17 (the forerunner of 17-1A), is now known to react with a 41 kDa glyc protein (see below).

35 Herlyn *et al.*, J. Clin. Immunol. 2:135 (1982), describes the

detection of a circulating colorectal carcinoma (CRC)-associated mAb developed against a membrane antigen of the SW116 cell 52a, which recognize a monosialoganglioside antigen (Magnan Science 212:55 (1981)), reacted with cells of 8 of 12 CRC 15 the cells of one gastric carcinoma and one pancreatic carcinoma reacted with four of six CRC cultures and with gastric tumor binding of mAbs 19-9 and 52a to tumor cells was inhibited by serum. However, CRC sera inhibited binding less frequently patients with pancreatic or gastric tumors.

10 Girardet et al., J. Immunol. 136:1497-1503 (1981) against human colon carcinomas. The L-D1 mAb reacted with a glycoprotein, believed to be the same antigen as that defined (Herlyn et al., 1979, supra). The L-C5 mAb precipitated protein molecular weights of 43, 45, 47 and 53 kDa from LoVo colon cancer. D1 did react with cervical carcinoma lines, while L-C5 reacted with carcinoma lines. Their binding to pancreatic carcinomas was:

15 Greiner et al., Science 235:895-898 (1987), described which reacts with a 90 kDa glycoprotein allegedly found in breast carcinomas and more than 90% of colon carcinomas.

20 Sakamoto et al., U.S. Patent 4,579,827 (4/1/86) describes a number of different approaches. None of these mAbs is shown to have a human colon carcinoma-associated antigen that is a protein of approximately 100 kDa molecular weight, distinguishing these antibodies from those of the present invention (described below). Furthermore, none of these mAbs have the degree of colon tumor specificity of the mAbs of the present application.

25 Delaloye et al., J. Clin. Invest. 77:301 (1986) describes a mAb specific for carcinoembryonic antigen (CEA) to detect carcinoma in vivo using <sup>123</sup>I-labelled fragments and single-photon emission tomography. The mAb described bears no relation to the mAb of the present invention.

30 Douillard et al., Hybridoma 5, Suppl. 1:S139 (1986) describes 17-1A and its cytotoxic properties to gastrointestinal adenocarcinoma in vitro. 17-1A was used with some degree of success in immunotherapy.

This mAb is said to recognize a 38-41 kDa protein and has a broad range of reactivity and lack of colon tumor specificity, clearly distinguishing it from the antibodies of the present invention.

Scannon *et al.*, U.S. Patent 4,590,071 (5/20/86), discloses mAbs specific for melanoma antigens conjugated to toxic proteins such as ricin A chain and the use of these compositions to treat melanoma. There is no disclosure directed to colon tumor antigens or antibodies and their uses.

The relatively pure antigen preparation containing the immunogenic colon carcinoma membrane antigens to which the mAbs of the present invention are directed was originally described by Hollinshead *et al.*, Science 177:887-889 (1972).

The clinical evaluation of the above antigen preparation, including a description of its immunogenicity and potential for enhancing patient survival through stimulation of specific active immunity, was described by Hollinshead *et al.*, 1985, supra.

The work of the present inventors leading to the present invention is briefly described in an abstract by Tsang *et al.*, "Monoclonal Antibodies to Human Colon Carcinoma Associated Antigens," Intl. Symp. Biotech. in Clin. Med., Rome, Italy, April 13-15, 1987. This reference was made available to the public less than one year before the filing of the ultimate parent application (U.S. Serial No. 07/176,337) for the present application.

#### SUMMARY OF THE INVENTION

The present inventors have produced murine mAbs and mouse-human chimeric antibodies specific for colon carcinoma-associated antigens which were known to be immunogenic in humans. These antigens, isolated in the inventors' laboratory, are unique among the previously described colon cancer antigens in that (1) the epitopes recognized by the mAbs are of the protein and not the carbohydrate component of tumor-associated glycoproteins; (2) the antigens are not expressed in normal tissues; (3) the antigens are tumor-specific, being present in malignancies of colon, breast, and ovarian cancer; (4) the antigens are immunogenic in humans, having the capability of enhancing host anti-tumor immunity by both cellular as well as humoral responses, thus improving survival in cancer patients; and (5) the immunogenicity in humans is

specific, in that only colon, breast and ovarian cancer patients, but not patients with other forms of cancer, show evidence of specific in vivo or in vitro immunological reactivity to the antigens.

The mAbs and chimeric antibodies of the present invention are useful for diagnosis or therapy of colon, breast, and ovarian carcinoma, for example by imaging metastatic tumors, by delivering cytotoxic agents to the tumors, and by activating host effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) to directly kill tumor cells.

The present invention is thus directed to a monoclonal antibody specific for a human colon carcinoma-associated protein antigen wherein the antigen is specifically immunogenic in humans, and the antigen is not detectable on normal human tissues or on human carcinoma cells other than colon, breast and ovarian carcinoma cells. Also included are antigen-binding fragments or derivatives of the antibody.

The present invention is also directed to a chimeric antibody specific for a human colon carcinoma-associated protein antigen wherein the antigen is not detectable on normal human tissues or on human carcinoma cells other than colon carcinoma cells.

In one embodiment, the antibody is specific for a CCAA which is a protein having a molecular weight of about 61 kilodaltons. In another embodiment, the antibody is specific for a CCAA which is a protein having a molecular weight of about 72 kilodaltons. In a preferred embodiment, the antibody is the mouse monoclonal antibody 33.28 or 31.1 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by 33.28 or 31.1. In another preferred embodiment, the antibody is a mouse/human chimeric antibody Chi #1 that binds specifically to the same colon carcinoma-associated epitope as that bound by 31.1.

The present invention is also directed to the above antibody immobilized on a solid phase.

The present invention includes the above antibody detectably labelled, for example, with a radiolabel.

In additional embodiments, the above antibody is conjugated to a cytotoxic radionuclide, a cytotoxic drug, or a cytotoxic protein.

In yet another embodiment, the present invention is directed to

monoclonal antibodies against the above antibodies, monoclonal antibodies.

In a further embodiment, the present invention provides generation monoclonal antibodies, i.e., monoclonal antibodies which are directed to the above second generation monoclonal antibodies.

The present invention is also directed to carcinoma-associated antigens which are unique in that the antigens recognized by the mAbs are of the protein and not the tumor-associated glycoproteins; (2) the antigens are found in tissues; (3) the antigens are tumor-specific, being found in malignancies of colon, breast and ovarian cancer; (4) immunogenic in humans, having the capability of enhancing the immune system, thus improving survival in cancer patients; (5) the immunogenicity in humans is specific, in that only cancer patients, but not patients with other forms of cancer, show specific in vivo or in vitro immunological reactivity.

To date, all other purified antigens that have been tested failed to elicit both a cellular and a humoral response.

The present invention also provides a pharmaceutical composition useful for the immunotherapy of colon, breast and ovarian cancer, comprising an antigen-binding fragment, antibody, fragment or derivative, as above, conjugated to a radionuclide, a cytotoxic drug, or a cytotoxic protein, or an excipient.

The present invention includes an immunoassay method for detecting a colon carcinoma-associated antigen capable of reacting with murine monoclonal antibody x Chi #1, comprising:

- contacting the sample with an antibody;
- detecting the antigen by detecting the antibody.

In another embodiment, the invention provides a method for detecting a colon carcinoma-associated antigen in a subject, comprising:

- contacting the detectably labelled antibody with the subject; and
- detecting the antigen.

The present invention also includes a method for

carrying a colon carcinoma-associated antigen, comprising:

- (a) delivering to the cells an antibody as above, and a cytotoxic effector agent; and
- (b) allowing the killing to occur.

5 The effector agent may be complement, or effector cells active in ADCC. Alternatively, antibodies labelled conjugated with a cytotoxic radionuclide, drug or protein may be used directly.

10 The present invention is further directed to a method of treating a subject suspected of having a colon, breast and ovarian carcinoma bearing an antigen which is capable of binding to the 33.28 or 31.1 monoclonal antibody, or Chi #1 chimeric antibody comprising administering to the subject an effective dose of a pharmaceutical composition as described above.

.5 Also provided is a method for producing an immunogenic composition useful for clinical immunotherapy of colon, breast, and ovarian carcinoma, comprising:

- (a) preparing a membrane extract of a tumor or cell line bearing an antigen which is capable of binding to the 33.28 or 31.1 monoclonal antibody or Chi #1 antibody; and
- (b) isolating the antigen by affinity purification using an antibody as described above,

:0 thereby producing the immunogenic composition.

In another embodiment, the present invention is directed to the use of the above antigen to produce a vaccine.

15 The present invention also includes a method of detecting and diagnosing colon, breast and ovarian cancer by staining monoclonal antibody or chimeric antibody bound to the above-described human colon carcinoma-associated antigen.

In another embodiment, the present invention includes a kit for selectively characterizing colon, breast, and ovarian carcinomas.

0

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a tracing showing an HPLC elution profile of the Hllinshead "vaccine," a partially purified preparation of colon carcinoma cell membranes.

fragments lack the Fc fragment of intact antibody, clear mo circulation, and may have less non-specific tissue binding antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)). It produced from intact antibodies using methods well kn wn in example by proteolytic cleavage with enzymes such as papain fragments) or pepsin (to produce  $F(ab')_2$  fragments).

A "derivative" of an antibody contains additi ns not normally a part of the protein. Covalent modifications included within the scope of this invention. Such modifcat introduced into the molecule by reacting targeted amino acid antibody with an organic derivatizing agent that is capable selected side chains or terminal residues. For exAMPL , der bifunctional agents, well-known in the art, is useful for cr antibody or fragment to a water-insoluble support matrix or macromolecular carriers.

By "vaccine" is meant an agent used to stimulate of a living organism so that immunological protection against caused by an infectious agent is provided. Administration contemplated by the present invention to the patient may be standard techniques. These include oral ingestion, intestin broncho-nasal spraying. Other methods of administration, suc injection, that allow the carrier microbe to reach the human bloodstream may be acceptable when the carrier microbe is une

The antibodies of the present invention are novel the first known mAbs and chimeric antibody specific for CCAA antigens are known to be immunogenic in humans. That is, the recognized by the antibodies of the present invention induce in patients with colon, breast, and ovarian cancer, but not i individuals, such as patients with other types of cancer. Th of these antigens is expressed chiefly as cell-mediated immun either by assay of delayed cutaneous hypersensitivity *in vivo* or by various *in vitro* assays of specific lymphocyte reactivi lymphocyte proliferation or lymphocyte migration inhibition a general principles of immunogenicity and description of vari specific immun 1 gical reactivity, see: Roitt, I., *Essential*

Ed., Blackwell Scientific Publications, Oxford (1988); Roitt, I. *et al.*, Immunology, C.V. Mosby Co., St. Louis, MO (1985); Klein, J., Immunology, Blackwell Scientific Publications, Inc., Cambridge, MA (1990); Klein, J., Immunology: The Science of Self-Nonself Discrimination, John Wiley & Sons, New York, NY (1982); Paterson, P.Y., Textbook of Immunopathology, Grune and Stratton, New York, (1986), which are hereby incorporated by reference.

In a preferred embodiment, the antibody of the present invention is a murine mAb designated 33.28. In another preferred embodiment, the antibody is a murine mAb designated 31.1. In yet another embodiment the antibody is a chimeric antibody which recognizes an epitope recognized by 33.28. In another embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by 31.1.

The chimeric antibodies of the invention comprise individual chimeric heavy (H) and light (L) immunoglobulin chains. The chimeric H chain comprises an antigen-binding region derived from the H chain of a non-human antibody specific for the epitope recognized by 33.28 or 31.1, which is linked to at least a portion of a human H chain C region ( $C_H$ ).

A preferred chimeric L chain comprises an antigen-binding region derived from the L chain of either the 33.28 or 31.1 mAb, linked to at least a portion of a human L chain C region ( $C_L$ ).

Alternatively, a preferred chimeric H chain comprises an antigen-binding region derived from the L chain of either the 33.28 or 31.1 mAb, linked to at least a portion of a human L chain C region ( $c_H$ ).

As used herein, the term "antigen-binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer ( $H_2L_2$ ) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a

$C_H$  region that aggregates (e.g., from an IgM H chain, or  $\mu$  chain).

The invention also provides for "derivatives" of the monoclonal or chimeric antibodies, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from prokaryotic or eukaryotic hosts, as described herein by recombinant means. Alternatively, the fragments and derivatives may be produced by chemical means, such as proteolytic cleavage of intact immunoglobulin molecules, or other chemical modifications or derivatizations known in the art. Such derivatized moieties may improve the solubility, absorption, biological half-life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the antibody protein. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Hack Publishing Co., Easton, PA (1980).

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different V region binding specificity can be prepared by appropriate association of the individual polypeptide chains, as taught, for example by Sears *et al.*, Proc. Natl. Acad. Sci. USA 72:353-357 (1975). With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives) and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or derivative.

Murine hybridomas which produce mAb specific for CCAA, such as the 33.28 and 31.1 mAbs of the present invention, are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against the CCAA.

Mice may be immunized with crude or semi-purified preparations containing the antigens of interest, such as, for example, the Hollinshead "vaccine," which is a partially purified membrane preparation of colon-carcinoma cells (Hollinshead *et al.*, *supra*). To immunize the mice, a variety of different conventional protocols may be followed. For example, mice may

receive primary and boosting immunizations of antigen.

The cell fusions are accomplished by standard techniques known to those skilled in the field of immunology (Kohler and Milstein, Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110; Hart and Campbell, A., "Monoclonal Antibody Technology," In: Immunobiology: Biochemistry and Molecular Biology, Volume 13 (Burd et al., eds. pp. 365-367, Elsevier, Amsterdam (1984); Kennett *et al.*, Monoclonal Antibodies: Production and Purification, Meth. Immunol. Meth. 35:1-21 (1980); Galfre, G. *et al.*, Monoclonal Antibodies, Meth. Immunol. Meth. 36:1-21 (1981); Goding, J.W. 1987, Monoclonal Antibodies: Principles and Practice, Academic Press, London, 1987).

Fusion partner cell lines and methods for hybridomas and screening for mAbs are well known in the art. See, supra; Kawamoto, T. *et al.*, Meth. Enzymol. 121:26-35 (1983); J.F. *et al.*, J. Immunol. 123:1548-1550 (1979); Kilmarx, P.H. *et al.*, J. Immunol. 138:576-582 (1982); Kohler, G. *et al.*, Eur. J. Immunol. 12:576-582 (1982); Lane, D.P. *et al.*, J. Immunol. Meth. 47:303-307 (1981); J. Immunol. Meth. 87:193-196 (1986); Pontecorvo, G., Proc. Natl. Acad. Sci. USA 72:400 (1975); Sharrow, J., *et al.*, Proc. Natl. Acad. Sci. USA 75:3032 (1978); Shulman, M. *et al.*, Nature 276:269-270 (1978); Springer, M. *et al.*, Technology in the Biosciences and Medicine, Plenum Press, New York (1982); Taggart, R.T. *et al.*, Science 219:1228-1230 (1982)).

The mAbs of the present invention may be produced in large quantities by injecting hybridoma cells secreting the mAb into the peritoneal cavity of mice and, after appropriate time, harvesting the fluid which contains a high titer of the mAb, and isolating the mAb. Alternatively, the mAbs may be produced by culturing the hybridoma cells and isolating the secreted mAb from the cell culture supernatant.

Human genes which encode the C regions of the mAbs of the present invention are derived from cells which produce human immunoglobulins. The human C<sub>H</sub> region gene encodes the known classes or isotypes of human H chains, including IgG, IgA, IgM, IgD, and IgE. Since the H chain isotype is responsible for the various biological properties of the antibody, the choice of C<sub>H</sub> region will be guided by the desired functions, such as complement fixation, opsonization, and activity in neutralizing viruses.

cellular cytotoxicity (ADCC). Preferably, the  $C_H$  region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), and  $\mu$  (IgM).

The human  $C_L$  region can be derived from either human L chain isotype, kappa or lambda.

5 Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains and the  
10 five classes of H chains. Chimeric antibody fragments, such as  $F(ab')_2$  and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding the H chain portion of an  $F(ab')_2$  fragment would include DNA sequences encoding the  $CH_1$  domain and hinge region of the H chain, followed by a translational stop codon  
15 to yield the truncated molecule.

Generally, the chimeric antibodies of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a CCAA-specific antibody, preferably non-human, most preferably 33.28 or 31.1, and joining these DNA segments to DNA segments encoding human  $C_H$  and  $C_L$  regions to produce chimeric immunoglobulin-encoding genes.  
20

Thus, in a preferred embodiment, a fused gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region. This fusion can be accomplished by the polymerase chain reaction, as  
25 reported by Fernando *et al.*, Miami Symp. Short Reports 3: 88, 1993.

The DNA encoding the antibody-binding region may be genomic DNA or cDNA. A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use  
30 of cDNA for the construction of chimeric immunoglobulin genes, as reported by Liu *et al.*, Proc. Natl. Acad. Sci., USA 84:3439 (1987) and J. Immunology 139:3521 (1987), which references are hereby incorporated by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired  
35 protein. The use of cDNA sequences is advantageous over genomic sequences

introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

5 Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

0 Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human  $C_H$  or  $C_L$  chain sequence having appropriate restriction sites engineered so that any  $V_H$  or  $V_L$  chain sequence with appropriate cohesive ends can be easily inserted therein. 5 Human  $C_H$  or  $C_L$  chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

) A chimeric mouse-human antibody will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human  $C_H$  region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

; Gene expression elements useful for the expression of cDNA genes include: (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, H. *et al.*, *Mol. Cell. Biol.* 3:280 (1983)), Rous sarcoma virus LTR (Gorman, C. *et al.*, *Proc. Natl. Acad. Sci., USA* 79:6777 (1982)), and Moloney murine leukemia virus LTR (Grosschedl, R. *et al.*, *Cell* 41:885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayama *et al.*, *supra*); and (c) polyadenylation sites such as in SV40 (Okayama *et al.*, *supra*).

Immunoglobulin cDNA genes may be expressed as described by Liu *et al.*, *supra*, and W idle *et al.*, *Gene* 51:21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain

Examples of selectable genes of use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gene from Tn5 (designated neo).  
5 Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis  
10 by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain  
20 vectors.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell.  
25 Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. A particularly preferred recipient cell is the Ig-non-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.  
30

The expression vector carrying a chimeric antibody construct of the present invention may be introduced into an appropriate host cell by any of a  
35

variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and  
5 microprojectile bombardment (Johnston *et al.*, Science 240:1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter *et al.*, Proc. Natl. Acad. Sci. USA 81:7161 (1984); Yoshikawa, K. *et al.*, Jpn. J. Cancer Res. 77:1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated.  
10 Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6 $\mu$ g/ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield  
15 transfection frequencies of about  $10^{-5}$  to about  $10^{-4}$  for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol.

The chimeric immunoglobulin genes of the present invention can also  
20 be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, *et al.*, 11th International Conference on Yeast, Genetics and Molecular Biology, Montpellier, France, September 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of chimeric H and L chain proteins and assembled chimeric antibodies. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycosidic enzymes produced in large  
35

quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches may be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, D.M., ed., DNA Cloning, Vol. II, pp. 45-66, IRL Press, 1985).

Bacterial strains may also be utilized as hosts for the production of antibody molecules or antibody fragments described by this invention. E. coli K12 strains such as E. coli W3110 (ATCC 27325), and other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species may be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches may be taken for evaluating the expression plasmids for the production of chimeric antibodies or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, D.M., ed., DNA Cloning, Vol. I, IRL Press, 1985).

Other preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which may be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned H and L chain genes in mammalian cells (see Glover, D.M., ed., DNA Cloning, Vol. II, pp. 143-238, IRL Press, 1985). Different approaches can be followed to obtain complete  $H_2L_2$  antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete trimeric  $H_2L_2$  antibodies. The

co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells may be transfected first with a 5 plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing H<sub>2</sub>L<sub>2</sub> molecules via either route could be transfected with plasmids encoding additional copies of H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines 10 with enhanced properties, such as higher production of assembled H<sub>2</sub>L<sub>2</sub> antibody molecules or enhanced stability of the transfected cell lines.

In addition to mAbs or chimeric antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for V region epitopes of the mAb antibody or chimeric antibody of the invention. An 15 anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for CCAA, such as 33.28, is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the Id antibody with the Id antibody 20 or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the 25 original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, the mAbs or chimeric antibodies of the present invention may be used to induce anti-Id antibodies in suitable animals, such as 30 BALB/c mice. Spleen cells from such immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for 35 a CCAA epitope.

The antibodies of the present invention, including their antigen-binding fragments and derivatives, have a multitude of uses relating to the diagnosis, monitoring and therapy of colon, breast, and ovarian cancer. Such uses are summarized in Schliom, J., Canc. Res., 46:3225-3238 (1986), which is hereby incorporated by reference.

In diagnosis, the antibodies may be used in immunoassays (described below) to screen body fluids, such as serum, sputum, effusions, urine, cerebrospinal fluid, and the like, for the presence of CCAA. The antibodies may be used for scanning or radioimaging, when labelled with an appropriate radiolabel, to detect primary or metastatic foci of tumor cells. Furthermore, the antibodies are useful in lymphoscintigraphy to detect lymph node involvement in the disease.

The antibodies of the present invention are also useful for immunopathological analysis, such as the differential diagnosis of tumor type, the subclassification of the tumor based on its expression of CCAA. Such determinations would be important in assessment of metastatic potential, predicted responses to therapy and prognosis.

In particular, because of the specificity of the mAbs and chimeric antibodies of the present invention, they may permit the definition of defining subpopulations of tumor cells among the heterogeneous cells present in a growing tumor. These antibodies could therefore be used in the typing and cross-matching of the tumor cell "lines" comprising the tumor by means of flow cytometry, both at the time of surgery and prior to therapy. An analysis of the tumor cell subpopulations with the antibodies of this invention, and a battery of additional mAbs, is used to define (a) which antigen preparation would be the most appropriate for specific active immunotherapy, (b) which mAb or chimeric antibody would be efficacious for ADCC, and (c) which antibody or combination of mAbs should be used for imaging the patient at a later date in search for recurrent or metastatic tumors.

In addition to their diagnostic utility, the antibodies of the present invention are useful for monitoring the progression of disease by screening body fluids for CCAA, radioimaging of tumor, or the detection of occult metastasis through aspiration cytology, lymph node or bone marrow biopsy, or cytology of body fluids.

A summary of the ways in which the antibodies of the present

invention may be used therapeutically includes direct cytotoxicity by the antibody, either mediated by complement (CDC) or by effector cells (ADCC), conjugated to anti-tumor drugs, toxins, radionuclides. The antibodies can be used for ex vivo removal of tumor cells from the circulation or from bone marrow.

Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

By the term "treating" is intended the administering to subjects of the antibodies of the present invention or a fragment or derivative thereof for purposes which may include prevention, amelioration, or cure of colon, breast, and ovarian cancer.

The pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. Amounts and regimens for the administration of antibodies, their fragments or derivatives can be determined readily by those with ordinary skill in the clinical art of treating colon, breast, and ovarian cancer and related disease.

For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all compositions wherein the antibody, fragment or derivative is contained in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. The effective dose is a function of the individual chimeric or monoclonal antibody, the presence and nature of a conjugated therapeutic agent (see below), the patient and his clinical status, and can vary from about 10 ng/kg body weight to about 100

10 mg/kg body weight. The preferred dosages comprise 0.1 to 10 mg/kg body weight.

In addition to the pharmacologically active compounds, the new pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound(s), together with the excipient.

Preparations of the antibody, fragment or derivative of the present invention for parenteral administration, such as in detectably labelled form for imaging or in a free or conjugated form for therapy, include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propyleneglycol, polyethyleneglycol, vegetable oil such as olive oil, and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media, parenteral vehicles including sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. See, generally, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Co., Easton, PA, 1980.

In particular, the antibodies, fragments and derivatives of the present invention are useful for treating a subject having or developing colon, breast, and ovarian adenocarcinoma. Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative, or a conjugate thereof.

The antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate ADCC and/or CDC against cells having CCAA associated with their surface. For these activities, either an endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized.

The antibodies of this invention, their fragments, and derivatives can be used therapeutically as immun conjugates (see for review: Dillman,

R.O., Ann. Int. Med. **111**:592-603 (1989)). They can be coupled to cytotoxic proteins, including, but not limited to, Ricin-A, Pseudomonas toxin, Diphtheria toxin, and tumor necrosis factor. Toxins conjugated to antibodies or other ligands are known in the art (see, for example, Olsnes, S. et al., Immunol. Today **10**:291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

The antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, diagnostic radionuclides and cytotoxic agents such as cytotoxic radionuclides, drugs and proteins. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include <sup>212</sup>Bi, <sup>131</sup>I, <sup>186</sup>Re, and <sup>90</sup>Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A.G., et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed., Macmillan Publishing Co., 1985.

The antibodies of this invention may be advantageously utilized in coordination with other monoclonal or chimeric antibodies, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies, fragments, or derivatives of this invention, attached to a solid support, can be used to remove soluble colon carcinoma-associated antigens from fluids or tissue or cell extracts. In a preferred embodiment, they are used to remove soluble tumor antigens from blood or blood plasma products. In another preferred embodiment, the antibodies are advantageously used in extracorporeal immunoabsorbent devices, which are known in the art (see, for example, Seminars in Hematology, V 1. 26 (2 Suppl. 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating CCAA (free or

in immune complexes), of CCAA-bearing cells, following which the fluid is returned to the body. This immunoadsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, D.S. et al., *J. Immunol.* 117:1971-1975 (1976).

5 The present invention also provides the above antibodies, fragments and derivatives, detectably labelled, as described below.

The antibodies of the present invention are useful for immunoassays which detect or quantitate CCAA or cells bearing CCAA in a sample. Such an immunoassay typically comprises incubating a biological sample in the presence 0 of a detectably labelled antibody of the present invention capable of identifying the tumor antigen, and detecting the labelled antibody which is bound in a sample.

Thus, in this aspect of the invention, a biological sample may be treated with nitrocellulose, or other solid support or carrier which is capable 5 of immobilizing cells, cell particles or soluble proteins or glycoproteins. The support may then be washed with suitable buffers followed by treatment with the detectably labelled antibody of the present invention. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support may then be detected 0 by conventional means.

By "solid phase support" or "carrier" is intended any support capable of binding antigen or antibodies. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or 5 insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to CCAA or the antibody specific for CCAA. Thus, the support configuration may be spherical, as in a bead, or cylindrical, ) as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody may be determined

according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the antibody of the present invention can be detectably labelled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). This enzyme, when subsequently exposed to its substrate, will react with the substrate generating a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. In an alternate embodiment, the enzyme is used to label a binding partner for the antibody of the invention. Such a binding partner may be an antibody against the constant or variable region of the antibody of the invention, such as a heterologous anti-mouse immunoglobulin antibody. Alternatively, the binding partner may be a non-antibody protein capable of binding to the antibody of the present invention, such as staphylococcal protein A, or streptococcal protein G.

Enzymes which can be used to detectably label the CCAA-specific antibodies of the present invention, or the binding partners for these antibodies, include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, gluc se oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glycoamylase and acetylcholinesterase.

By radioactively labelling the antibody of the present invention or the binding partner, it is possible to detect CCAA through the use of a radioimmunoassay (RIA) (see, for example, Work, T.S. *et al.*, Laboratory Techniques and Biochemistry in Molecular Biology, North Holland Publishing Company, N.Y. (1978)). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are well known in the art.

It is also possible to label the antibodies or binding partners with a fluorescent compound. When the fluorescently labelled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling

compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin,  $\alpha$ -phthaldehyde and fluorescamine.

The antibodies can also be detectably labelled using fluorescence-emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The antibodies of the present invention also can be detectably labelled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labelled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labelling compounds are luminol, isoluminol, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody, fragment or derivative of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems, in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labelling are luciferin, luciferase and aequorin.

Detection of the antibody, fragment or derivative may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

In situ detection may be accomplished by removing a histological specimen from a patient, and providing the labelled antibody, or the unlabelled antibody plus a labelled binding partner to such a specimen. Through the use of such a procedure, it is possible to determine not only the presence of the antigen but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified

in order to achieve such in situ detection. Such methods include, for example, immunohistochemical staining procedures. In a preferred embodiment, an avidin-biotin immunoperoxidase staining system can be used, and a kit utilizing this system is also contemplated.

5           The kit employing mAbs or chimeric antibodies of the present invention can be used for immunohistochemical evaluation of colon, breast, and ovarian carcinoma. Indications for tissue study are to evaluate subpopulations of tumor cells that express the antigens defined by mAbs 31.1 and 33.28.

10           The colon kit is comprised of the reagents necessary for immunohistochemical analysis as follows:

- a)       mAbs 31.1, 33.28 or mouse/human chimeric antibody Chi #1, and the mAb for carcinoembryonic antigen (CEA), the latter representing the standard monoclonal used for colon tissue immunohistochemistry;
- b)       reagents for immunoperoxidase (blocking reagent) in the form of, for example, goat serum; and secondary antibody, such as, for example, goat anti-mouse antibody;
- c)       immunoperoxidase; and
- d)       reagents to produce the brown coloration.

20           Similar kits can be employed for the immunohistochemical analysis of breast and ovarian carcinoma.

25           The immunoperoxidase technique to be employed is that of Sternberger. The primary antibody (mAb or chimeric antibody) serves as an antigen which can bind more than one secondary antibody. The secondary antibodies form a "bridge" between the primary antibody and the horseradish peroxidase-antiperoxidase complexes.

30           The kit contemplated herein can be used to study fully developed colon, carcinoma, polyps intransformation to define the extent of malignant transformation, benign polyps to see if a site of transformation has been missed and inflammatory bowel disease to evaluate any sites of undetected

transformation. Similar kits can be employed to study breast and ovarian carcinomas.

Another kit similar to the above kit is also contemplated which uses all five mAbs to colon carcinoma in order to evaluate all subpopulations 5 of tumors and as such has the capability to type and cross-match the lesions.

The antibody, fragment or derivative of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabelled antibody (or fragment of antibody), is bound to a solid support that is 10 insoluble in the fluid being tested and a quantity of detectably labelled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labelled antibody.

Typical, and preferred, immunometric assays include "forward" 15 assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the tumor antigen from the sample by formation of a binary solid phase antibody-CCAA complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted tumor antigen, if any, and then contacted 20 with the solution containing an unknown quantity of labelled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labelled antibody to complex with the CCAA bound to the solid support through the unlabelled antibody, the solid support is washed a second time to remove the unreacted labelled antibody. This type of forward sandwich 25 assay may be a simple "yes/no" assay to determine whether CCAA is present or may be made quantitative by comparing the measure of labelled antibody with that obtained for a standard sample containing known quantities of the antigen. Such "two-site" or "sandwich" assays are described by Wide (Radioimmune Assay Method, Kirkham, ed., E. & S. Livingstone, Edinburgh, 1970, pp. 199-206).

Other type of "sandwich" assays, which may also be useful with 0 CCAA, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labelled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to 5 remove the residue of fluid sample and uncomplexed labelled antibody. The

presence of labelled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labelled antibody to the fluid sample, followed by the addition of unlabelled antibody bound to a solid support after a suitable incubation period, is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labelled antibody. The determination of labelled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes may be used to construct a sensitive three-site immunoradiometric assay.

For purposes of in vivo imaging of colon, breast, and ovarian cancer using the antibodies of the present invention, there are many different labels and methods of labelling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes, paramagnetic isotopes, and compounds which can be imaged by positron emission tomography (PET). Those of ordinary skill in the art will know of other suitable labels for binding to the antibodies used in the invention, or will be able to ascertain such, using routine experiments. Furthermore, the binding of these labels to the antibody can be done using standard techniques common to those of ordinary skill in the art.

For diagnostic in vivo imaging, the type of detection instrument available is a major factor in selecting a given radionuclide. The radionuclide chosen must have a type of decay which is detectable by a given type of instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention.

Another important factor in selecting a diagnostic radionuclide for in vivo imaging is that the half-life of a radionuclide be long enough so that it is still detectable at the time of maximum uptake by the target issue, but short enough so that deleterious radiation of the host is minimized. In one preferred embodiment, a radionuclide used for in vivo imaging does not emit particles, but produces a large number of photons in a 140-200 keV range, which may be readily detected by conventional gamma cameras.

For in vivo diagnosis, radionuclides may be bound to the antibody

either directly or indirectly by using an intermediary functional group. Intermediary functional groups which are often used to bind radioisotopes which exist as metallic ions to the antibodies are the chelating agents, diethylene triamine pentaacetic acid (DTFA) and ethylene diamine tetraacetic acid (EDTA). Examples of metallic ions which can be bound to the antibodies of the present invention are  $^{99m}\text{Tc}$ ,  $^{123}\text{I}$ ,  $^{111}\text{In}$ ,  $^{131}\text{I}$ ,  $^{97}\text{Ru}$ ,  $^{67}\text{Cu}$ ,  $^{67}\text{Ga}$ ,  $^{125}\text{I}$ ,  $^{68}\text{Ga}$ ,  $^{72}\text{As}$ ,  $^{89}\text{Zr}$ , and  $^{201}\text{Tl}$ .

The specifically exemplified mAbs 33.28 and 31.1, and the chimeric antibody Chi #1 may be used to facilitate the production of additional mAbs which bind the same or immunologically cross-reactive colon carcinoma-associated antigens. First, these antibodies may be conjugated to a chromatographic support, and used to immunopurify colon carcinoma-associated antigens. These purified antigens, in turn, may be used to stimulate an immune response in suitable animals. Secondly, spleen cells from the responsive animals may be fused to immortalizing cells, and the resulting hybridomas screened for secretion of antibodies which bind to the purified antigen and/or whose binding to colon carcinoma-associated antigen is competitively inhibited by antibody 33.28 or 31.1, or chimeric antibody Chi #1.

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

#### EXAMPLE I

##### Preparation and Characterization of the Colon Carcinoma-Associated Antigen (CCAA)

The antigenic preparation was obtained from pooled colon carcinoma membranes according to the method described by Hollinshead *et al.*, Cancer **56**:480 (1985). This antigenic material was purified to the extent that the membrane fractions were free of HL-A antigens and were separated from much of the non-immunogenic glycoprotein fractions. In its final form the antigenic preparation was shown to be immunogenic in a specific manner in humans as evidenced by its capability of eliciting a delayed hypersensitivity reaction only in patients with active col n, breast, and ovary carcinoma.

Tumor cell suspensions in saline were prepared from fresh operating room specimens. Single cell suspensions, obtained by conventional means, were centrifuged for 10 minutes at about 400 x g and the supernatant was retained. The cell pellet was resuspended and recentrifuged. The membrane material was examined by electron microscopy to assure that only membrane material (and no intact cells) was present, and the protein content was measured by the Lowry method.

The membrane material was next subjected to sequential low frequency sonication and resuspended as a soluble pool of membrane proteins. The soluble sonicates were separated by gel filtration on Sephadex-6200. Fractions of 2 ml were collected and the absorbance profile at 220 and 280  $\mu$ m was recorded. Fractions comprising individual protein peaks were pooled, and the pools were concentrated by Diaflo ultrafiltration. Sephadex-G200 fractions IB and IIA, as defined by Hollinshead *et al.*, (*supra*), were further purified by gradient polyacrylamide gel electrophoresis (PAGE). The fractions were tested for their ability to elicit positive delayed cutaneous hypersensitivity reactions in patients with colon carcinoma. Those fractions with immunogenic activity were said to contain colon carcinoma-associated antigens (CCAA) and were employed as immunogens and screening agents in the preparation of the mAbs.

By gradient PAGE, a double-banded antigen distinct from that of carcinoembryonic antigen (Gold, P. *et al.*, *J. Exp. Med.* 122:467-481 (1965); Hollinshead, A. *et al.*, *Cancer* 56:480 (1985)) was identified and isolated. The bands comprising this antigen migrated 6.3 and 6.6 cm. distant from tracking dye. Biochemical analysis of the antigen proved that it was glycoprotein. The molecular weight of the antigen was estimated based on the electrophoretic mobility of transferrin (6.4-6.5 cm) which has a molecular weight of 76.5 kDa.

#### EXAMPLE II

##### Preparation and Screening of Monoclonal Antibodies

Monoclonal antibodies (mAbs) against human colon carcinoma-associated antigens (CCAA) were obtained by the production and cloning of hybrids resulting from the fusion of mouse myeloma cells Sp2/0-Agl4 with spleen cells from BALB/c mice which had been immunized with the CCAA described above.

Five hybrid clones were established, as described below, and designated as 31.2, 31.1, 77, 33.23 and 33.28. All five mAbs reacted strongly with the CCAA and with two colon carcinoma cell lines (SW480 and SW620) when assayed by ELISA. Two of the mAbs, 31.1. and 33.28, were studied in greatest detail.

5 A. Immunization and Cell Fusion

BALB/c mice were immunized by intraperitoneal injection of 50 µg of the CCAA described above emulsified in complete Freund's adjuvant, as described by Hollinshead in clinical trials (Hollinshead *et al.*, *supra*). Ten days later the mice received an intravenous booster injection of the same amount of CCAA in saline. Mice were sacrificed three days later and their spleen cells obtained. Cell fusion was performed by incubation  $5 \times 10^7$  mouse spleen cells with  $10^7$  sP2/O-Agl4 myeloma cells in 40% polyethylene glycol (MW-1500)

10 B. Screening of Hybrid Clones

15 An enzyme-linked immunosorbent assay (ELISA), described by Tsang *et al.*, *JNCI* 77:1175 (1986), was used for the detection of hybridoma clones producing antibodies specific for the CCAA. CCAA (100 ng/well) was immobilized on polystyrene microplates. Antibodies present in the test supernatants were allowed to bind to the immobilized antigens. The presence of the bound murine 20 mAbs was detected with peroxidase-conjugated second antibodies, specific for mouse immunoglobulins, followed by the chromogenic substrate for peroxidase, O-phenyldiamine. Wells showing color reactions yielding Absorbances  $\geq 0.500$  units were scored as positive. Negative controls gave values of 0.01 to 0.09 units.

25 Hybridoma wells scoring as positive by ELISA were further screened by indirect immunofluorescence, using various tumor cells and normal cells as identified in Table 1, below. All of the tumor cell lines were obtained from the ATCC. Cells were incubated with hybridoma culture supernatants at an appropriate dilution (1:2) in phosphate buffered saline (PBS) for 1 hour at 30 4°C. The cells were washed and incubated with a fluorescein-labelled goat anti-mouse immunoglobulin antibody. The cells were then washed three times with PBS and examined by fluorescence microscopy. The results appear in Table 1.

Table 1

INDIRECT IMMUNOFLUORESCENCE REACTIVITY OF ANTI-COLON  
CARCINOMA-ASSOCIATED ANTIGEN(S) (COAA) MoAbS WITH HUMAN  
CULTURE CELLS<sup>A</sup>

---

	CELLS	REACTIVITY OF MoAbS <sup>B</sup>				
		31.2	77	31.1	33.28	33.23
5	<u>TUMOR LINES</u>					
	SW948(COL)	-	+	-	-	-
	HCT116(COL)	-	-	-	+	-
	WIDR(COL)	+	+	+	+	+
	COLO320(COL)	+	+	+	-	-
10	HS619(COL)	-	-	-	-	-
	HS853(COL)	-	-	-	-	-
	CACO-2(COL)	+	+	-	-	-
	SK-CO-1(COL)	+	+	-	+	+
	HT-29(COL)	+	+	-	+	+
15	SW1116(COL)	+	-	-	+	+
	SW480(COL)	+	+	+	+	+
	SW620(COL)	+	+	+	+	+
	231(BR)	-	-	-	-	-
	CAMA-1(BR)	-	-	-	-	-
20	PAN-1(PAN)	-	-	+	-	-
	MIA(PAN)	-	-	+	-	-
	HS766T(PAN)	-	-	-	-	-
	M-14(MEL)	-	-	-	-	-
	HT1080(FIB)	-	-	-	-	-
25	LM(OS)	-	-	-	-	-
	TE-85(OS)	-	-	-	-	-

NORMAL SKIN

FIBROBLAST

BONE MARROW

CELL

5      NORMAL HUMAN

PBMC

---

- A.      CULTURE SUPERNATANT WAS DILUTED 1:2 WITH PBS.
- B.      POSITIVE (+) AND NEGATIVE (-) REACTIVITIES WERE DEFINED BY INTENSITY OF THE MEMBRANE FLUORESCENCE AS COMPARED TO THE BACKGROUND.
- 10     C.      COL: COLON CARCINOMA; BR: BREAST CARCINOMA;  
PAN: PANCREATIC CARCINOMA; FIB: FIBROSOMCOMA.

EXAMPLE IIIAnalysis of Monoclonal Antibodies and their Reactivity

The anti-CCAA mAbs produced and detected as above were also tested for reactivity with fresh human tissue. Cryostat sections of the tissue types listed in Table 2, below, were fixed with 3.5% formaldehyde in PBS and then washed three times with PBS. For indirect immunofluorescence studies, the sections were incubated with the mAbs and then stained with a fluorescein-labelled second antibody as above.

As is shown in Table 2, mAbs 31.1 and 33.28 were highly specific for colon carcinoma cells. This indicates that an antigen (CCAA) which was highly specific for colon carcinoma and, furthermore, was immunogenic in colon carcinoma patients (positive DH reactions), and served as a successful immunogen in mice for the devnd phycoerythrin excitation was used. Trigger regions were established by examining cells by forward versus 90° light scatter. As shown in Table 4, both 31.1 and 33.28 bound to colon carcinoma cells; neither mAb bound significantly to PBMC.

Table 2

Indirect Immunofluorescence of Anti-CCAA Mabs with  
Flesh Human Tissues\*

20

<u>Tissues</u>	<u>33.28</u>	<u>31.1</u>
<b>Tumor</b>		
Colon Carcinoma	3/3	3/3
Pancreatic Carcinoma	0/2	0/2
Melanoma	0/2	0/1
Breast Carcinoma	0/2	0/1

Normal

Placenta	0/1	0/1
Liver	0/1	0/1
Colon	0/3	0/3
Spleen	0/1	0/1
5 Thymus	0/1	0/1
Muscle	0/1	0/1

10 a. Ascitic fluid was diluted 1:50 with PBS. Cryostat sections (4-6 µM thick) were fixed with 3.5% formaldehyde in PBS for 10 minutes and then washed three times in PBS. Sections were stored at -70°C unless used immediately.

Results are expressed as number of positive/negative of tissue tested.

15 Table 3 shows the results of an immunoabsorption analysis of the Mabs. Three colon carcinoma cell lines (HT-29, WIDR and SW 620) and an osteosarcoma cell line (LM) were used to absorb fluorescein isothiocyanate (FITC)-conjugated Mabs 31.1 or 33.28. Ascites fluid from mice in which the hybridomas were growing, diluted 1:50, was added to the absorbing cells. The mixtures were incubated for 1 hr at 4°C. Either  $2 \times 10^7$  cells (Table 3, Part 20 A) or  $10^4$  cells (Table 3, Part 5) were used to absorb the antibodies (Table 3). The osteosarcoma cell line did not absorb out 31.1 or 33.28 activity, while the colon carcinoma cell lines did.

Table 3

Immunoabsorption Analysis of mAbs

Absorbing Cells

mAb	<u>HT-29</u>		<u>WIDR</u>		<u>SW620</u>		<u>LM</u>	
	A	B	A	B	A	B	A	B

30 33.28 - + - + - + + +

31.1	-	+	-	+	-	+	+	+
------	---	---	---	---	---	---	---	---

Cytofluorometric analysis was used to measure the binding of 31.1, 33.28 and a control mAb to HT29, WiDr and SW480 tumor cells and to peripheral blood mononuclear cells (PBMCs). An Ortho Spectrum III Cytofluorograph, equipped with an argon laser capable of fluorescein and phycoerythrin excitation was used. Trigger regions were established by examining cells by forward versus 90° light scatter. As shown in Table 4, both 31.1 and 33.28 bound to colon carcinoma cells; neither mAb bound significantly to PBMC.

10

Table 4

## SUMMARY OF CYTOFLUOROMETRIC ANALYSIS

% of cells stained with mAb:

	<u>Cells 33.28</u>	<u>31.1</u>	<u>Control</u>	
15	HT29	51.0	53.9	9.2
	WiDr	21.0	ND	8.1
	SW480 37.0	32.0	3.8	
	PBMC	2.1	2.1	ND

20        The heavy and light chain isotypes of the mAbs were determined by immunodiffusion. The 31.1 mAb was found to be an IgG1 with a kappa light chain. The 33.28 mAb was found to be an IgG2a with a kappa light chain (Table 5). This is in strong contrast to the prior art mAb 19.9 (Herlyn, M. *et al.*, *J. Biol. Chem.* 257:14365-14369 (1982)) which is of the IgG1 class (Herlyn, D. *et al.*, *Proc. Natl. Acad. Sci. USA* 79:4761-4765 (1982)). Importantly, antibodies of the IgG2a class are expected to be more useful for immunotherapeutic purposes (Colcher, D. *et al.*, *Proc. Natl. Acad. Sci. USA* 78:3199-3203 (1981)). Although the 19.9 mAb has reactivity to colon tumors, it was derived by immunization with pancreatic carcinoma antigens, and is therefore cross-reactive with colon. This is analogous to the situation with the B72.3 mAb ([citation]), which is a colon-reactive antibody obtained by

immunization with breast cancer tissue.

Table 5

## Isotyping of Monoclonal Antibodies

5

	Culture Supernatant	IgG1	IgG2a	IgG2b	IgM	Light Chains	
						Kappa	Lambda
0	31.2	-	+	-	-	+	-
	31.1	+	-	-	-	+	-
	77	-	-	+	-	+	-
	33.23	-	+	-	-	+	-
	33.28	-	+	-	-	+	-

EXAMPLE IVCharacterization of the Colon Carcinoma-Associated Antigen

The molecular mass of the antigens to which the above mAbs bound was determined by Western blot analysis using soluble protein extracted from colon carcinoma cell lines SW480 and SW620. The 33.28 and 31.1 mAbs reacted with molecules having apparent molecular weights of 61.1 kDa and 72 kDa, respectively, from both of these cell lines. These mAbs did not react with material from human PBMCs or from human tumor cell lines of other histologic types in Western blot analysis.

In order to define better the specificity of the mAbs of the present invention for the immunizing CCAA and to establish whether the mAbs reacted with an immunogenic component of the cell membrane preparation which has been used in clinical immunotherapy trials (Hlinish ad *et al.*, *supra*), the original immunogenic preparation described above was performed by high performance liquid chromatography (HPLC).

The analysis revealed 4 distinct peaks, each of which was tested for immune reactivity (elicitation of DH) in patients with colon carcinoma by skin test (Figure 1). Among the 10 patients with colon carcinoma tested only the material in peak #4 induced a cutaneous DH reaction. The peak #4 antigen 5 was found to react with mAb 33.28, while mAb 31.1 reacted with peak #3, the next most prominent peak.

The references cited above are all incorporated by reference herein.

#### EXAMPLE V

10 Affinity Purification of Colon Carcinoma-Associated Antigen

The mAb 33.28 was used in affinity chromatography to isolate antigen extracted from cells of the HT-29 line. Five mg of purified 33.28 IgG was coupled to CNBr-activated sepharose 4B. The column was pre-eluted with 0.05M diethylamine, pH 11.5, and then equilibrated with 0.14M NaCl/0.01M Tris (pH 8.0). CCAA preparation was applied to the column, and the column was eluted with 0.05M diethylamine, pH 11.5. The eluted fractions were neutralized by the addition of 1M Tris-HCl, pH 8.0.

15 The material bound and eluted from the 33.28 affinity matrix was then subjected to HPLC. The eluted CCAA preparation was adjusted in starting buffer (0.01M sodium phosphate buffer, pH 7.0), applied to a Synchropak Wax weak anion exchange HPLC column (250 x 4.6 mm) and eluted with a gradient of 0 to 1M NaCl in 0.01M sodium-phosphate buffer, pH 6.0, at a flow rate of 1 ml/min. Anion exchange chromatography was performed using a Hewlett-Packard HPLC (HP 1090, Hewlett-Packard, Arondale, PA).

20 25 Results appear in Figure 2. The antigenic material derived from HT-29 cells isolated by mAb 33.28 gave a peak that matched peak #4 described above and had similar immunogenic activity in humans, indicating the utility of mAb 33.28 for isolation of a colon cancer preparation which is immunogenic for humans.

#### ADCC Activity of mAbs 33.28 and 31.1

In order to be therapeutically useful, a mAb specific for an immunogenic tumor antigen should have the following properties: (a) high tumor tissue specificity, (b) absence of cross-reactivity to normal human tissue, and (c) a biological activity associated with destruction of tumors, such as antibody-dependent cellular cytotoxicity (ADCC).

The ADCC activity of mAbs 33.28 and 31.1 was tested on the colon carcinoma line WiDR as target cell. The melanoma cell line, M-14, served as a specificity control. ADCC was assayed using a conventional 4 hr.  $^{51}\text{Cr}$  release assay using normal human PBMC as effector cells, and the results are shown as percent isotope release (% lysis) (Table 6). The background lysis was 8.3%. At an effector:target ratio of 100:1, mAb 33.28 caused 40.3% lysis of tumor cells, and 31.1 induced 51.8% lysis.

Table 6

## .5 ADCC Activity of mAbs 33.28 and 31.1

## % Lysis of Target Cells at E:T Ratios:

Antibody or Control	WiDR			M-14		
	25	50	100	25	50	100
33.28	23.1	40.3	45.3	6.9	8.4	9.0
31.1	14.3	26.7	51.8	7.5	6.4	8.7
OSA1	10.0	9.2	12.2	11.4	14.8	10.9
NMS	12.2	11.7	13.1	14.2	15.0	11.1
PBS	8.2	5.1	7.6	11.0	14.2	10.5

.5 ADCC was assayed by a 4 hr  $^{51}\text{Cr}$  release assay. Background  $^{51}\text{Cr}$  release was

8.3%. E:T Ratio indicates effector cell-to-target cell ratios. The mAb or serum was tested at a 1:100 dilution; OSAl--mAb to osteosarcoma associated antigens; NMS--normal mouse serum; WiDr--colon carcinoma cell line; M14--melanoma cell line.

5

---

#### EXAMPLE VII

##### Detection of Circulating CCAA with mAbs 33.28 and 31.1

The mAbs of the present invention were tested for their ability to detect circulating CCAA in 79 unknown serum samples (Table 7). The assay was based on the ability of the serum samples to inhibit binding of the mAb to the CCAA in ELISA. None of the 50 normal serum samples gave false positive results. Nine of the ten serum samples from patients with active colon carcinoma were positive. None of the sera from disease-free colon cancer patients one year post-resection were positive.

15

Table 7

##### Detection of Circulating Colon Carcinoma-Associated Antigen

##### No. of sera inhibiting binding of mAbs:

	DONOR <u>CONDITION</u>	No. of Samples	<u>33.28</u>		<u>31.1</u>	
			<15% (Neg)	>15% (Pos)	<15% (Neg)	>15% (Pos)
20	Colon Carcinoma	10	3	7	2	8
25	Colon (Resected) Carcinoma	4	4	0	4	0
	Breast Carcinoma	9	9	0	9	0
	Melanoma	5	5	0	5	0

43

**Prostate**

Cancer	1	1	0	1	0
Normal Serum	50	50	0	50	0

---

5      Colon carcinoma-associated antigens was detected by ELISA. 100  $\mu$ l of serum were used in each assay.

---

**EXAMPLE VIII****Comparison of Specificity with Other mAbs Reactive with Colon Tumors**

0      Further studies of tumor specificity were conducted using ELISA (Table 8). The mAb 31.1 was compared with CC49, a colorectal carcinoma-specific mAb purified from B-72.3, and a control mouse myeloma protein. 31.1 was shown to react with a narrower range of colorectal carcinomas than did 5      CC49. However, it had a higher degree of specificity, having lower or no cross-reactivity with stomach tumors or normal colon tissue.

TABLE 8

ELISA ON NORMAL AND TUMOR TISSUES USING MAbs 31.1, CC49 AND MOPC-21

Tissues	31.1	CC49	MOPC-21
<b>Colorectal Carcinomas</b>			
1. COCA2A	-	+++	-
2. COCA2	-	+++	-
3. COCA3	+	++	-
4. COCA4	+++	+++	-
5. G820	#	++	-

44

6.	G853	+++	++	-
7.	G817	+++	+++	-
8.	G781	-	-	-

Other Carcinomas

5	1.	Breast CA1	-	-
	2.	Breast CA2	-	-
	3.	Lung CA1	-	-
	4.	Lung CA2	-	-
	5.	Ovarian CAD106	-	-
10	6.	Ov CAS	-	-
	7.	Ov CAV5	-	-
	8.	Ov CAV45	-	-
	9.	Ov CAV43	-	-
	10.	Stomach CA14A	++	+++
15	11.	Stomach CA12A	-	+++
	12.	Stomach CA15A	-	-

Other Normal Tissues

20	1.	Endometrium E21	-	-
	2.	Endometrium EC19	-	-
	3.	Endometrium EC17	-	+
	4.	Endometrium EC18 (RBC)	-	-
	5.	Red blood cells 1	-	-
	6.	RBC 2	-	-
25	7.	RBC 3	-	-
	8.	RBC 4	-	-
	9.	RBC 5	-	-
	10.	RBC 6	-	-
	11.	RBC 7	-	-

	12.	RBC 8	-	-	-	-
	13.	RBC 9	-	-	-	-
	14.	RBC 10	-	-	-	-
	15.	RBC 11	-	-	-	-
5	16.	Granulocytes	-	-	-	-
	17.	385	-	-	-	-
	18.	386	-	-	-	-
	19.	Normal spleen 3	-	-	-	-
	20.	392 (N. Spleen)	-	-	-	-
10	21.	395 (N. Liver)	-	-	-	-
	22.	387 (N. Kidney)	-	-	-	-
	23.	398 (N. Spleen)	-	-	-	-
	24.	390 (N. Liver)	-	-	-	-
	25.	N. Spleen #1	-	-	-	-
15	26.	N. Spleen #2	-	-	-	-
	27.	800 (N. Colon)	-	++	-	-
	28.	N. Colon (GW)	-	++	-	-
	29.	N. Colon (Meloy)	-	-	-	-
	30.	N. Colon	-	-	-	-
20	31.	G1155B (N. Colon)	-	-	-	-
	32.	G1164B (N. Colon)	-	-	-	-
	33.	N. Colon	±	-	-	-
	34.	Normal Stomach A	-	-	-	-
	35.	N. Stomach B	-	-	-	-
25	36.	N. Stomach C	-	-	-	-
	37.	Normal Lung	-	-	-	-
	38.	Normal Liver	-	-	-	-

CC49 - NCI monoclonal antibody to colorectal carcinomas

MOPC-21 - negative control myeloma protein

All mon clonal antibodies were used at 40 ng/well, POGAM at 1:3000 dilution

EXAMPLE IXIn Vivo Localization of mAbs 33.28 and 31.1 to Tumors

The in vivo behavior of the mAbs of the present invention was examined by pharmacokinetic studies using  $^{125}\text{I}$ -labelled mAb and athymic nude mice bearing LS-174T colon tumor xenografts. Mice implanted with the A375 melanoma were employed as controls. The relative concentration of mAb in tumor as compared to adjacent normal tissue such as liver and spleen is represented by the radiolocalization index (concentration of radiolabelled material in tumor/concentration in surrounding tissue). The biodistribution of  $^{125}\text{I}$ -labelled 31.1 and 33.28 mAbs is shown in Table 9. Both mAbs were able to significantly concentrate within the tumor, compared to localization in normal tissues (spleen and liver). This selective accumulation was six-fold at 96 hours and about 12-fold at 168 hrs. The radiolocalization indices for both mAbs to tumor as compared to blood, liver and spleen are shown in Figure 3 and Figure 4.

Table 9Biodistribution of  $^{125}\text{I}$ -mAbs in Tumor-Bearing Athymic Nude Mice

Tissue	96 hours		168 hours	
	LS174T	A375	LS174T	A375
<b>A. mAb 31.1</b>				
Blood	7.30	NA	4.67	4.42
Tumor	21.92	NA	25.43	2.91
Liver	3.74	NA	2.16	1.24
Spleen	3.68	NA	2.41	1.32

B. mAb 33.28

Blood	7.81	NA	5.58	3.95
Tumor	13.12	NA	15.50	2.24
Liver	2.55	NA	1.74	1.68
5 Spleen	2.31	NA	1.70	1.92

Results are expressed in % injected dose/gram of tissue.

LS174T - colon carcinoma; A375 - melanoma.

.0

EXAMPLE XImmunohistochemical Studies

The ability to detect tumor markers in the serum, image the related neoplastic process and define the cell population of that neoplasm by immunohistochemistry depends on the ability of specific mAbs to selectively characterize a tumor population.

The mAbs 31.1 and 33.28 were tested in more than 50 colon carcinomas by means of immunoperoxidase staining. They have been found to be highly reactive with the colon neoplasm and did not interact with the adjacent normal tissues. When polyps were evaluated, the wholly benign lesions such as villo-tubular adenomas showed no reactivity. Villous adenomas undergoing transformation reacted only at the site of malignancy. When similar tissues were evaluated using the more common carbohydrate-antigen derived mAbs, normal adjacent colon tissue reacted equally with the neoplastic portion of the specimen. With the 31.1 and 33.28 mAbs, each appeared to stain different cell populations within the tumor. This suggests that surface antigens representing different oncogene products were being defined.

.5

.0

5

EXAMPLE XI

Selective Binding to Subpopulations of Epithelial Cells in  
Paraffin-Embedded Benign and Malignant Mammary Lesions

Forty-one formalin-fixed, paraffin-embedded benign and malignant breast specimens were studied with mAbs 31.1 and 33.28, using the avidin-biotin staining method. Without enzymatic pretreatment, positive staining of epithelium was observed on the cell surface and in the cytoplasm with both antibodies. 7/21 (33%) duct carcinogens were positive with mAb 31.1 as were 5/20 (25%) samples of benign breast disease. 10/21 (48%) duct carcinomas were positive with mAb 33.28 together with 7/20 (35%) specimens of benign mammary disease. 10 to 75% of the cell population was positively stained. These results indicate that the antigens defined by mAbs 31.1 and 33.28 are expressed in a select group of women with breast disease and would be useful for diagnosis of said disease.

EXAMPLE XII

15      Selective Binding to Subpopulations of Epithelial Cells in Fresh Frozen Benign and Malignant Ovarian Tumors

Fresh frozen tissue biopsies obtained from twenty-one ovarian tumors subjected to immunocytochemical analysis were studied with mAbs 31.1 and 33.28 using the avidin biotin indirect immunoperoxidase assay. Focal positive staining was observed in 4/7 papillary mucous, 1/1 mucinous and 1/2 endometroid adenocarcinoma. None of the nonepithelial ovarian tumors stained positive using these monoclonal antibodies. These results indicate that the antigens as defined by mAbs 31.1 and 33.28 are expressed in a select group of woman with ovarian cancer and would be useful for diagnosis of said disease.

25

EXAMPLE XIII

Immunoreactivity of Human Carcinoma Tissues and  
Cell Lines with mAbs 31.1 and 33.28

The mAbs 31.1 and 33.28 were used to screen a panel of cell lines including colon aden carcinoma, lymphoma, leukemia and neuroblastoma lines. 30 Using the avidin-biotin immunoperoxidase staining system, the mAbs

31.1 and 33.28 were shown to strongly bind to colon adenocarcinoma cell lines WIDR and HT-29. Immunoreactions were not observed with KG1-a, HL-60, Molt-3 and JUKRAT cell lines. Both antibodies reacted weakly with one lymphoma line (JY). The mAb 33.28 reacted weakly with one leukemic line (K562) and a neuroblastoma line (U87.MG). These results confirm and extend previous flow cytometry and immunofluorescent results in which it has been reported that strong binding reactions were observed with these mAbs with colon adenocarcinoma cell lines and reactions were not observed with other tumor cell lines. Using flow cytometry, mAb 31.1 reacted with 85% of HT-29 and WIDR 10 colon carcinoma cells but not with SKBR-3 breast cancer cells.

Both antibodies were extensively shown to bind distinctively to colon carcinoma tissues (mAb 33.28 - 84%, mAb 31.1 - 64%), and not to normal tissues or malignant tissues including neuroblastoma tissues (0/3), lymphomas (0/3) and leukemic infiltrates (0/3) tested. These results suggest that these 15 antibodies can serve as a useful research tool in evaluating tumor markers in cancer and cell biology research.

#### EXAMPLE XIV

##### Expression and Characterization of Chimeric Antibodies Against Human Colorectal Carcinoma-Associated Antigen

20 A chimeric mouse/human heavy chain gene was constructed by splicing the exon of the 31.1 antibody heavy chain variable region gene to the exon of the human gamma chain constant region gene using the polymerase chain reaction. Subsequently, the 31.1 chimeric gene was cloned into a retroviral expression vector pLgptCXII and transfected into the packaging cell line PA317. The 25 transfected cells (PA317H) were cultivated with another packaging cell line PA317L, which contained an irrelevant mouse/human chimeric light chain gene in retroviral expression vector pLneoCXII, and SP2/0-Agl4 cells. The transduced SP2/0-Agl4 cells yielded a complete chimeric antibody, Chi #1 which reacted with horse radish peroxidase -c conjugated IgG of goat anti-human IgG Fc in ELISA analyses, which indicated that the constant region of Chi #1 was human. 10 Cyt fluorometry analysis indicated that Chi #1 stained human colorectal

carcinoma cell lines HT-29 and LS174T but not a human lung carcinoma cell line A-427. Antibody-dependent cell-mediated cytotoxicity (ADCC) assay indicated that Chi #1 lysed LS174T cells. These results show that Chi #1 retained the antigen-binding specificity of the parental 31.1 mouse monoclonal antibody,  
5 suggesting the useful of this chimeric antibody in ascertaining prognosis of colon carcinoma.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing  
10 from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles  
15 of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A monoclonal antibody specific for a human colon carcinoma-associated protein antigen, wherein said antigen has the following characteristics:

- 5 (i) said monoclonal antibody recognizes epitopes of a protein component of said antigen, and not epitopes of a carbohydrate component of said antigen;
- (ii) said antigen is not detectable on normal human tissues;
- .0 (iii) said antigen is not detectable on human carcinoma cells other than colon carcinoma cells; and
- (iv) said antigen is specifically immunogenic in humans.

2. An antibody according to Claim 1 which is mouse monoclonal antibody 33.28 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by 33.28.

15 3. An antibody according to Claim 2 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 61.1 kilodaltons.

0 4. An antibody according to Claim 1 which is mouse monoclonal antibody 31.1 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

5 5. An antibody according to Claim 4 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 72 kilodaltons, wherein said colon carcinoma-associated antigen is a glycoprotein, the protein component having a molecular weight of 61.1 kilodaltons.

6. An antibody according to Claim 1 immobilized on a solid phase.

7. An antibody according to Claim 1 which is detectably labelled.

8. An antibody according to Claim 7 wherein said detectable label is a radiolabel.

9. An antibody according to Claim 1 conjugated to a cytotoxic radionuclide.

10. An antibody according to Claim 1 conjugated to a cytotoxic drug.

11. An antibody according to Claim 1 conjugated to a cytotoxic protein.

12. A pharmaceutical composition for the immunotherapy of colon, breast, and ovarian cancer in humans comprising an antibody according to Claim 9 in combination with a pharmaceutically acceptable carrier.

5 13. A pharmaceutical composition for the immunotherapy of colon, breast, and ovarian cancer in humans comprising a therapeutically effective amount of an antibody according to Claim 10 in combination with a pharmaceutically acceptable carrier.

10 14. A pharmaceutical composition for the immunotherapy of colon, breast, and ovarian cancer in humans comprising a therapeutically effective amount of an antibody according to Claim 11 in a pharmaceutically acceptable carrier.

15 15. A human colon carcinoma-associated protein antigen having the following characteristics:

(i) epitopes of a protein component of said antigen are capable of eliciting an antibody response in a mammalian host, but epitopes of a carbohydrate component of said antigen are not capable of eliciting said antibody response;

20 (ii) said antigen is not detectable on normal human tissues;

(iii) said antigen is not detectable on human carcinoma cells other than colon carcinoma cells; and

(iv) said antigen is specifically immunogenic in humans.

25 16. An antigen according to Claim 15 wherein said antigen is a protein having a molecular weight of about 61 kilodaltons.

17. An antigen according to Claim 15 wherein said antigen is a protein having a molecular weight of about 72 kilodaltons.

30 18. An antigen according to Claim 16 wherein said antigen binds specifically to mouse monoclonal antibody 33.28 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 33.28.

35 19. An antigen according to Claim 17 wherein said antigen binds specifically to mouse monoclonal antibody 31.1 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

20. A monoclonal antibody against the monoclonal antibody of  
Claim 1.

21. A monoclonal antibody against the monoclonal antibody of  
Claim 2.

5 22. A monoclonal antibody against the monoclonal antibody of  
Claim 3.

23. A monoclonal antibody against the monoclonal antibody of  
Claim 4.

10 24. A monoclonal antibody against the monoclonal antibody of  
Claim 5.

25. An immunoassay method for detecting a colon carcinoma-  
associated antigen capable of binding to mouse monoclonal antibody 33.28 in a  
sample comprising:

15 (a) contacting said sample with an antibody according to Claim  
1; and

(b) detecting said antigen by detecting the binding of the  
antibody.

20 26. An immunoassay method for detecting a colon carcinoma-  
associated antigen capable of binding to mouse monoclonal antibody 31.1 in a  
sample, comprising:

(a) contacting said sample with an antibody according to Claim  
1; and

(b) detecting said antigen by detecting the binding of the  
antibody.

25 27. An imaging method for detecting a colon carcinoma associated  
antigen in a subject, comprising:

(a) contacting the labelled antibody of Claim 7 with said  
animal; and

(b) detecting said antigen.

30 28. A method of killing cells carrying a colon carcinoma-  
associated antigen, comprising delivering to said cells an effective amount of  
an antibody according to Claim 1 and a cytotoxic effector agent.

29. A method according to Claim 28 wherein said effector agent  
is selected from the group consisting of complement and effector cells active  
35 in antibody-dependent cellular cytotoxicity.

30. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an antibody according to Claim 9.

5 31. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an antibody according to Claim 10.

32. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an antibody according to Claim 11.

10 33. A method of treating a subject suspected of having a colon carcinoma in humans bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, comprising administering to said subject an effective dose of the composition of Claim 12.

15 34. A method of treating a subject suspected of having a colon carcinoma bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, comprising administering to said subject an effective dose of the composition of Claim 13.

20 35. A method of treating a subject suspected of having a colon carcinoma bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, comprising administering to said subject an effective dose of the composition of Claim 14.

36. A method for producing an immunogenic composition useful for clinical immunotherapy of colon carcinoma in humans, comprising:

25 (a) preparing a membrane extract of a tumor or cell line bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1; and  
(b) isolating said antigen by affinity purification using an antibody according to Claim 6,

thereby producing said composition.

30 37. A vaccine for immunization against colon cancer in humans which comprises an effective amount of an antigen according to Claim 15 or active fragments thereof in association with a conventional vaccine carrier.

35 38. A vaccine for immunization against colon cancer in humans which comprises an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse

monoclonal antibody 33.28 or 31.1, in association with a conventional vaccine carrier.

39. A method for immunotherapy of colon cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of an antigen according to Claim 15 or active fragments thereof in association with a conventional vaccine carrier.

40. A method for immunotherapy of colon cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse monoclonal antibody 33.28 or 31.3, in association with a conventional vaccine carrier.

41. The vaccine according to Claim 37 wherein the dosage effective range is about 0.001-100 mg antigen/kg body weight.

42. The vaccine according to Claim 38 wherein the dosage effective range is about 0.001-100 mg antigen/kg body weight.

43. A method for diagnosing colon cancer in humans comprising:

- (a) removing a histological specimen from a patient suspected of having a colon carcinoma bearing an antigen according to Claim 15;
- (b) contacting the specimen with a monoclonal antibody capable of recognizing said antigen;
- (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex by the stain.

44. A method for diagnosing colon cancer in humans comprising:

- (a) removing a histological specimen from a patient suspected of having a colon carcinoma bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1;
- (b) contacting the specimen with mouse monoclonal antibody capable of recognizing said antigen;
- (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex.

45. The method of Claim 43 wherein the stain is an avidin-biotin immunoperoxidase stain.

46. The method of Claim 44 wherein the stain is an avidin-biotin

immunoperoxidase stain.

47. A kit for the immunohistochemical detection of colon carcinoma comprising:

- a) mouse monoclonal antibodies 31.1 and 33.28, and monoclonal antibody for carcinoembryonic antigen;
- b) reagents for immunoperoxidase and secondary antibody;
- c) immunoperoxidase; and
- d) colorizing reagents.

48. A kit for immunohistochemical detection of colon carcinoma comprising:

- a) mouse monoclonal antibodies 31.1, 31.2, 33.23, 33.28 and 77 and monoclonal antibody for carcinoembryonic antigen;
- b) reagents for immunoperoxidase and secondary antibody; and
- c) immunoperoxidase; and
- d) colorizing reagents.

49. A compartmentalized kit for the detection of an antigen according to Claim 15, said kit comprising: a first container adapted to contain an antibody to said antigen or an active component thereof, and a second container adapted to contain a second antibody to said antigen or an active component thereof, said second antibody being labeled with a reporter molecule capable of giving a detectable signal.

50. The kit according to Claim 49 wherein the reporter molecule is a radioisotope, an enzyme, a fluorescent molecule, a chemiluminescent molecule or a bioluminescent molecule.

51. The kit according to Claim 49 wherein the reporter molecule is an enzyme.

52. The kit according to Claim 49 wherein the kit further comprises a third container adapted to contain a substrate for the enzyme.

53. A monoclonal antibody specific for a human colon carcinoma-associated protein antigen, wherein said antigen has the following characteristics:

- (i) said monoclonal antibody recognizes epitopes of a protein component of said antigen, and not epitopes of a carbohydrate component of said antigen;
- (ii) said antigen is not detectable on normal human tissues;

- (iii) said antigen is not detectable on human carcinoma cells other than breast carcinoma cells; and
- (iv) said antigen is specifically immunogenic in humans.

5 54. An antibody according to Claim 53 which is mouse monoclonal antibody 33.28 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by 33.28.

.0 55. An antibody according to Claim 54 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 61.1 kilodaltons.

.5 56. An antibody according to Claim 53 which is mouse monoclonal antibody 31.1 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

.5 57. An antibody according to Claim 53 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 72 kilodaltons, wherein said colon carcinoma-associated antigen is a glycoprotein, the protein component having a molecular weight of 61.1 kilodaltons.

:0 58. A pharmaceutical composition for the immunotherapy of breast cancer in humans comprising a therapeutically effective amount of an antibody according to Claim 53 in combination with a pharmaceutically acceptable carrier.

:5 59. A human colon carcinoma-associated protein antigen having the following characteristics:

- (i) epitopes of a protein component of said antigen are capable of eliciting an antibody response in a mammalian host, but epitopes of a carbohydrate component of said antigen are not capable of eliciting said antibody response;
- (ii) said antigen is not detectable on normal human tissues;
- (iii) said antigen is not detectable on human carcinoma cells other than breast carcinoma cells; and
- (iv) said antigen is specifically immunogenic in humans.

0 60. An antigen according to Claim 59 wherein said antigen is a protein having a molecular weight of about 61 kil daltons.

5 61. An antigen according to Claim 59 wherein said antigen is a protein having a molecular weight of about 72 kilodaltons.

62. An antigen according to Claim 60 wherein said antigen binds specifically to mouse monoclonal antibody 33.28 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 33.28.

5 63. An antigen according to Claim 61 wherein said antigen binds specifically to mouse monoclonal antibody 31.1 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

10 64. A monoclonal antibody against the monoclonal antibody of Claim 53.

65. An immunoassay method for detecting a colon carcinoma-associated antigen capable of binding to mouse monoclonal antibody 33.28 in a sample comprising:

15 (a) contacting said sample with an antibody according to Claim 53; and  
(b) detecting said antigen by detecting the binding of the antibody.

66. An immunoassay method for detecting a colon carcinoma-associated antigen capable of binding to mouse monoclonal antibody 31.1 in a sample, comprising:

20 (a) contacting said sample with an antibody according to Claim 53; and  
(b) detecting said antigen by detecting the binding of the antibody.

25 67. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an effective amount of an antibody according to Claim 53 and a cytotoxic effector agent.

30 68. A method of treating a subject suspected of having a breast carcinoma in humans bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, comprising administering to said subject an effective dose of the composition of Claim 58.

69. A vaccine for immunization against breast cancer in humans which comprises an effective amount of an antigen according to Claim 59 or active fragments thereof in association with an adjuvant vaccine carrier.

35 70. A vaccine for immunization against breast cancer in humans

which comprises an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, in association with a conventional vaccine carrier.

5        71. A method for immunotherapy of breast cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of an antigen according to Claim 59 or active fragments thereof in association with a conventional vaccine carrier.

10      72. A method for immunotherapy of breast cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse monoclonal antibody 33.28 or 31.3, in association with a conventional vaccine carrier.

15      73. A method for diagnosing breast cancer in humans comprising:  
(a) removing a histological specimen from a patient suspected of having a breast carcinoma bearing an antigen according to Claim 59;  
(b) contacting the specimen with a monoclonal antibody capable of recognizing said antigen;  
20      (c) staining the specimen with an immunohistochemical stain; and  
(d) detecting the presence of the antigen-antibody complex by the stain.

25      74. A method for diagnosing breast cancer in humans comprising:  
(a) removing a histological specimen from a patient suspected of having a breast carcinoma bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1;  
(b) contacting the specimen with mouse monoclonal antibody capable of recognizing said antigen;  
30      (c) staining the specimen with an immunohistochemical stain; and  
(d) detecting the presence of the antigen-antibody complex.

75. A kit for the immunohistochemical detection of breast carcinoma comprising:

- a) mouse monoclonal antibodies 31.1 and 33.28, and monoclonal antibody for carcinoembryonic antigen;
- b) reagents for immunoperoxidase and secondary antibody;

- c) immunoperoxidase; and
- d) colorizing reagents.

76. A monoclonal antibody specific for a human colon carcinoma-associated protein antigen, wherein said antigen has the following characteristics:

- (i) said monoclonal antibody recognizes epitopes of a protein component of said antigen, and not epitopes of a carbohydrate component of said antigen;
- (ii) said antigen is not detectable on normal human tissues;
- (iii) said antigen is not detectable on human carcinoma cells other than ovarian carcinoma cells; and
- (iv) said antigen is specifically immunogenic in humans.

77. An antibody according to Claim 76 which is mouse monoclonal antibody 33.28 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by 33.28.

78. An antibody according to Claim 77 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 61.1 kilodaltons.

79. An antibody according to Claim 76 which is mouse monoclonal antibody 31.1 or an antibody which binds specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

80. An antibody according to Claim 79 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of about 72 kilodaltons, wherein said colon carcinoma-associated antigen is a glycoprotein, the protein component having a molecular weight of 61.1 kilodaltons.

81. A pharmaceutical composition for the immunotherapy of ovarian cancer in humans comprising a therapeutically effective amount of an antibody according to Claim 76 in combination with a pharmaceutically acceptable carrier.

82. A human colon carcinoma-associated protein antigen having the following characteristics:

- (i) epitopes of a protein component of said antigen are capable of eliciting an antibody response in a mammalian host, but epitopes of a carbohydrate component of said antigen are not capable of

eliciting said antibody response;

(ii) said antigen is not detectable on normal human tissues;

(iii) said antigen is not detectable on human carcinoma cells other than ovarian carcinoma cells; and

5 (iv) said antigen is specifically immunogenic in humans.

83. An antigen according to Claim 82 wherein said antigen is a protein having a molecular weight of about 61 kilodaltons.

84. An antigen according to Claim 82 wherein said antigen is a protein having a molecular weight of about 72 kilodaltons.

10 85. An antigen according to Claim 83 wherein said antigen binds specifically to mouse monoclonal antibody 33.28 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 33.28.

15 86. An antigen according to Claim 84 wherein said antigen binds specifically to mouse monoclonal antibody 31.1 or an antibody capable of binding specifically to the same colon carcinoma-associated epitope as that bound by mouse monoclonal antibody 31.1.

87. A monoclonal antibody against the monoclonal antibody of Claim 76.

20 88. An immunoassay method for detecting a colon carcinoma-associated antigen capable of binding to mouse monoclonal antibody 33.28 in a sample comprising:

(a) contacting said sample with an antibody according to Claim 76; and

25 (b) detecting said antigen by detecting the binding of the antibody.

89. An immunoassay method for detecting a colon carcinoma-associated antigen capable of binding to mouse monoclonal antibody 31.1 in a sample, comprising:

30 (a) contacting said sample with an antibody according to Claim 76; and

(b) detecting said antigen by detecting the binding of the antibody.

90. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an effective amount of

an antibody according to Claim 76 and a cytotoxic effector agent.

91. A method of treating a subject suspected of having an ovarian carcinoma in humans bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, comprising administering to said subject an effective dose of the composition of Claim 81.

92. A vaccine for immunization against ovarian cancer in humans which comprises an effective amount of an antigen according to Claim 82 or active fragments thereof in association with a conventional vaccine carrier.

93. A vaccine for immunization against ovarian cancer in humans which comprises an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse monoclonal antibody 33.28 or 31.1, in association with a conventional vaccine carrier.

94. A method for immunotherapy of ovarian cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of an antigen according to Claim 82 or active fragments thereof in association with a conventional vaccine carrier.

95. A method for immunotherapy of ovarian cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse monoclonal antibody 33.28 or 31.3, in association with a conventional vaccine carrier.

96. A method for diagnosing ovarian cancer in humans comprising:

- (a) removing a histological specimen from a patient suspected of having an ovarian carcinoma bearing an antigen according to Claim 82;
- (b) contacting the specimen with a monoclonal antibody capable of recognizing said antigen;
- (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex by the stain.

97. A method for diagnosing ovarian cancer in humans comprising:

- (a) removing a histological specimen from a patient suspected of having an ovarian carcinoma bearing an antigen which is capable of binding to mouse monoclonal antibody 33.28 or 31.1;
- 5 (b) contacting the specimen with mouse monoclonal antibody capable of recognizing said antigen;
- (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex.

98. A kit for the immunohistochemical detection of ovarian

10 carcinoma comprising:

- a) mouse monoclonal antibodies 31.1 and 33.28, and monoclonal antibody for carcinoembryonic antigen;
- b) reagents for immunoperoxidase and secondary antibody;
- c) immunoperoxidase; and
- d) colorizing reagents.

99. A chimeric antibody specific for a human colon carcinoma-associated protein antigen, wherein said antigen has the following characteristics:

- (i) said chimeric antibody recognizes epitopes of a protein component of said antigen, and not epitopes of a carbohydrate component of said antigen;
- (ii) said antigen is not detectable on normal human tissues;
- (iii) said antigen is not detectable on human carcinoma cells other than colon carcinoma cells; and
- (iv) said antigen is specifically immunogen in humans.

100. A chimeric antibody according to claim 99 which is a chimeric mouse/human antibody Chi #1.

101. A chimeric antibody according to claim 100 wherein said colon carcinoma-associated antigen is a protein having a molecular weight of 72 kilodalton.

102. A pharmaceutical composition for the immunotherapy of colon cancer in humans comprising a therapeutically effective amount of an antibody according to Claim 99 in combination with a pharmaceutically acceptable carrier.

103. An antigen according to claim 101 wherein said antigen binds specifically to chimeric antibody Chi #1, or a chimeric antibody capable of binding specifically to the same colon-carcinoma-associated epitope as that bound by chimeric antibody Chi #1.

5 104. A monoclonal antibody against the chimeric antibody antibody of Claim 99.

105. An immunoassay method for detecting a colon carcinoma-associated antigen capable of binding to mouse/human chimeric antibody Chi #1 in a sample comprising:

10 (a) contacting said sample with an antibody according to Claim 99; and  
(b) detecting said antigen by detecting the binding of the antibody.

15 106. A method of killing cells carrying a colon carcinoma-associated antigen, comprising delivering to said cells an effective amount of an antibody according to Claim 99 and a cytotoxic effector agent.

20 107. A method of treating a subject suspected of having a colon carcinoma in humans bearing an antigen which is capable of binding to mouse/human chimeric antibody Chi #1, comprising administering to said subject an effective dose of the composition of Claim 102.

108. A vaccine for immunization against colon cancer in humans which comprises an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse/human chimeric antibody Chi #1, in association with a conventional vaccine carrier.

25 109. A method for immunotherapy of colon cancer in humans comprising administering to humans in need thereof a vaccine having an effective amount of human colon carcinoma-associated antigen or active fragments thereof which is capable of binding to mouse/human chimeric antibody Chi #1, in association with a conventional vaccine carrier.

110. A method for diagnosing colon cancer in humans comprising:

- (a) removing a histological specimen from a patient suspected of having a colon carcinoma bearing an antigen according to Claim 15;
- 5 (b) contacting the specimen with a chimeric antibody capable of recognizing said antigen;
- (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex by the stain.

10 111. A method for diagnosing colon cancer in humans comprising:

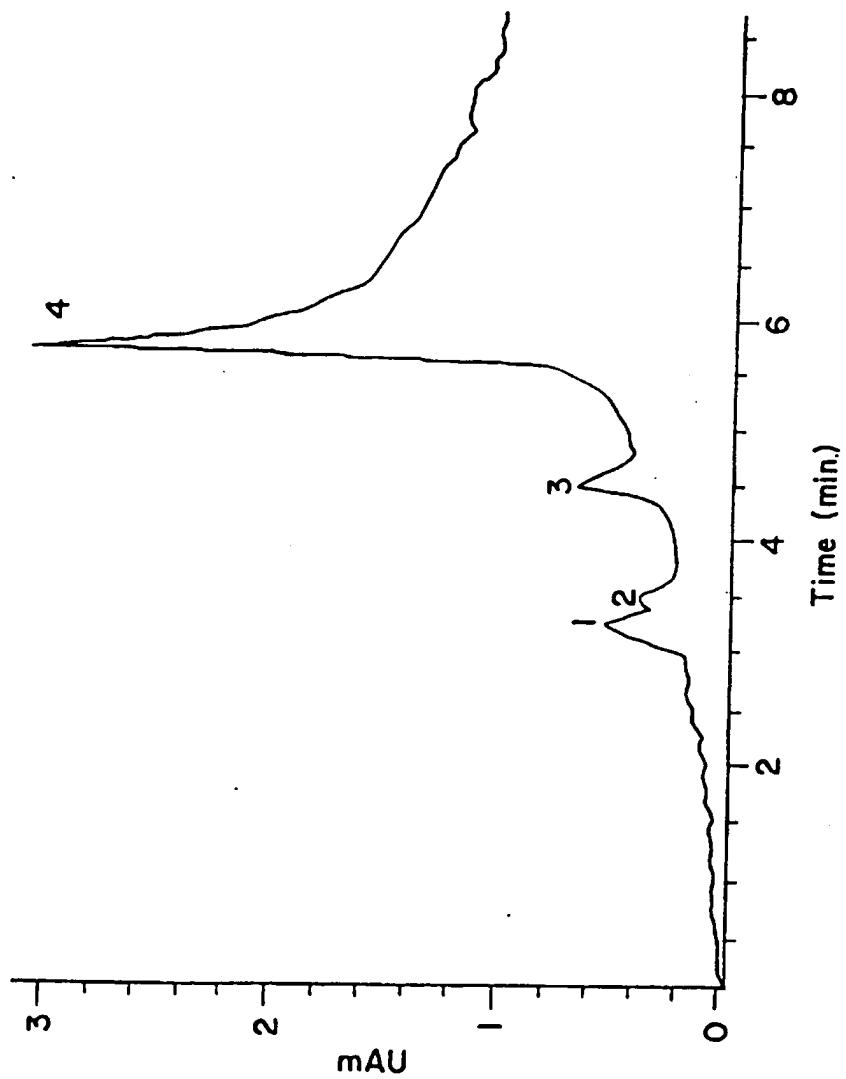
- (a) removing a histological specimen from a patient suspected of having a colon carcinoma bearing an antigen which is capable of binding to mouse/human chimeric antibody Chi #1;
- (b) contacting the specimen with mouse/human chimeric antibody capable of recognizing said antigen;
- 15 (c) staining the specimen with an immunohistochemical stain; and
- (d) detecting the presence of the antigen-antibody complex.

112. A kit for the immunohistochemical detection of colon carcinoma comprising:

- 20 a) mouse/human chimeric antibodies Chi #1, and chimeric antibody for carcinoembryonic antigen;
- b) reagents for immunoperoxidase and secondary antibody;
- c) immunoperoxidase; and
- d) colorizing reagents.

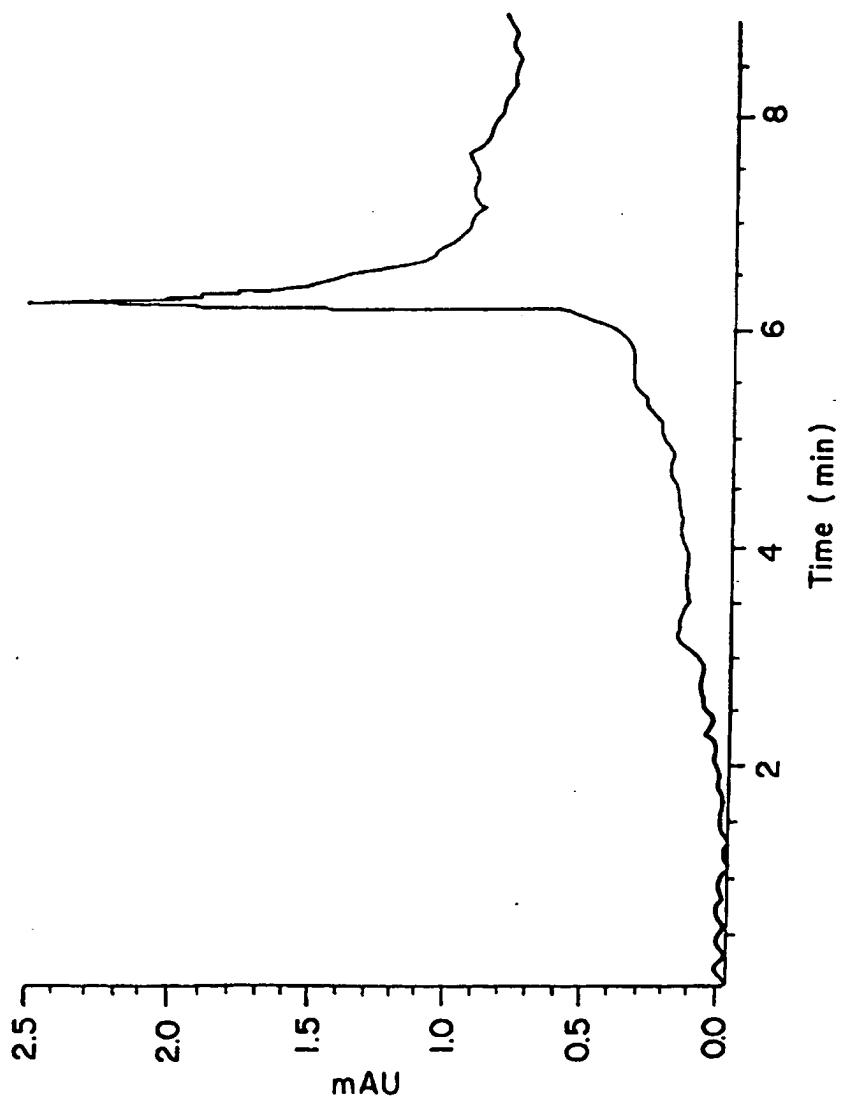
1 / 4

FIG. I



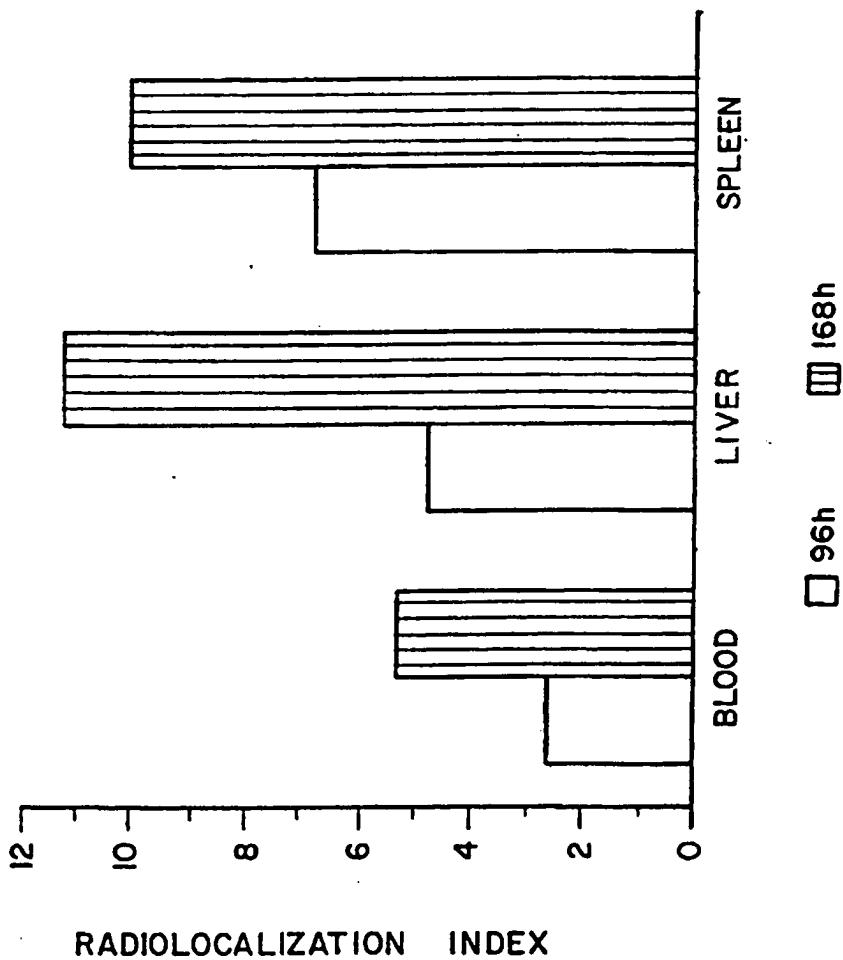
2 / 4

FIG. 2



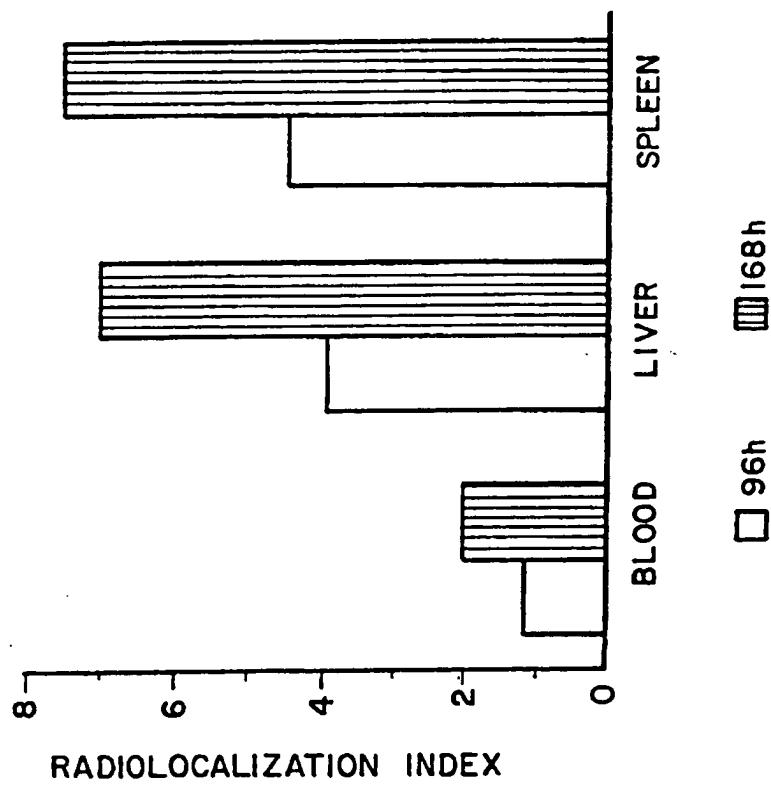
3 / 4

FIG. 3



4 / 4

FIG. 4



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/11554

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.8, 387.3, 350; 424/1.49, 155.1, 277.1; 435/7.23, 172.2, 70.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, Medline, Embase, Cancerlit, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Antibody, Immunoconjugates, and Radiopharmaceuticals, Volume 4, Number 4, Issued 1991, M. Arlen et al., "Monoclonal Antibodies to Immunoreactive Tumor Associated Antigen (TAA) from Human Colon Carcinoma", pages 895-905.	1-32, 43-58, 65-67, 73-81, 87-90, 96-102, 104-106, 110-112
X	FASEB, Volume J5, Number 4, Issued April 21, 1991, D. L. Xu, "Construction of a Mouse/Human Chimeric Light Chain From Murine Monoclonal Antibody 33.28 Against Colorectal Carcinoma-Associated Antigens", page A2109.	1-14, 20-24, 53-58, 64, 76-81, 87, 99-102, 104

 Further documents are listed in the continuation of Box C. See patent family annex.

- \* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

Date of the actual completion of the international search

13 DECEMBER 1995

Date of mailing of the international search report

24 JAN 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer  
*Susan A. Loring*  
SUSAN A. LORING

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/11554

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB, Volume J2, Number 5, Issued 1988, Kwong Y. Tsang et al., "Colon Carcinoma Associated Antigens Characterized by Monoclonal Antibodies", Abstract 6815.	1-14, 20-26, 36, 43-58, 64-66, 73-81, 87-89, 96-102, 104, 105, 110-112
Y	Cancer Research, Volume 46, Issued April 1986, Norman R. Hughes et al., "Antigen Expression in Normal and Neoplastic Colonic Mucosa: Three Tissue-specific Antigens Using Monoclonal Antibodies to Isolated Colonic Glands", pages 2164-2171, see entire document.	1-112
X	Proceedings of the American Association for Cancer Research, Volume 34, Issued March 1993, J. Wolf et al., "Monoclonal Antibody 31.1 May Differentiate Indolent From Aggressive Colon Cancer", page 452, Abstract # 2695.	1, 4-14, 20, 23-24, 53, 56-58, 64, 76, 79-81, 87
Y	Clinical Biochemistry, Volume 25, Issued 1992, Masahide Kuroki et al., "Reaction Profiles of Seven Enzyme Immunoassay Kits for Carcinoembryonic Antigen (CEA) Analyzed with Purified Preparations of CEA and Related Normal Antigens", pages 29-35, see entire document.	25-32, 43-52, 65-67, 73-75, 88-90, 96-98, 105-106, 110-112
X	Molecular Biology of the Cell, Volume 4, Supplement, Issued December 11, 1993, J. Wolf et al., "Immunoreactivity of Cell Lines and Colon Carcinomas with Monoclonal Antibodies PCA 31.1 and PCA 33.28", page 334A, Abstract # 1940.	1-14, 20-24, 53-58, 64, 76-81, 87, 99-102, 104
Y	Cancer Immunology and Immunotherapy, Volume 35, Issued 1992, Esther Hurwitz et al., "Immunotargeting of Daunomycin to Localized and Metastatic Human Colon Adenocarcinoma in Athymic Mice", pages 186-192, see entire document.	1-14, 20-24, 27, 53-58, 64, 76-81, 87, 99-102, 104
X	Journal of Surgical Oncology, Volume 54, Issued 1993, Myron Arlen et al., "Monoclonal Antibodies and Their Role in Modulation of the Immune Response", pages 103-108, see entire document.	1-112
X	Proceedings of the American Association for Cancer Research, Volume 31, Issued March 1990, A. H. Bartal et al., "Monoclonal Antibody Defining an Antigen Present within a Purified Tumor Membrane Vaccine", page 260, Abstract # 1537.	1, 4-14, 20, 23-24, 53, 56-58, 64, 76, 79-81, 87

**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/US95/11554**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/US95/11554**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

C07K 16/00, 16/30, 16/18, 14/00; A61K 39/395, 41/00, 49/00, 51/00; G01N 33/574; C12N 15/02; C12P 21/08

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

S30/388.8, 387.3, 350; 424/1.49, 155.1, 277.1; 435/7.23, 172.2, 70.21

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-14, 20-26, 36, 43-58, 64-66, 73-81, 87-89, 96-102, 104-105, and 110-112, drawn to a monoclonal antibody, pharmaceutical composition, immunoassay and kit, method for producing an immunogenic composition useful for immunotherapy and a chimeric antibody.

Group II, claim(s) 15-19, 59-63, 82-86 and 103, drawn to a human colon carcinoma-associated protein antigen.

Group III, claim 27, drawn to an imaging method.

Group IV, claims 28-35, 67-68, 90-91, and 106-107, drawn to a method for killing cells and a method for treating by administering an effective amount of an antibody.

Group V, claims 37-38, 41-42, 69-70, and 92-93, drawn to a vaccine comprising a human colon carcinoma associated protein antigen.

Group VI, claims 39-40, 71-72, 94-95, 108-109, drawn to a method of immunotherapy by administering a vaccine comprising a human colon carcinoma associated protein antigen.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I comprises an antibody, pharmaceutical composition, immunoassay (method of use), method of producing an immunogenic composition useful for immunotherapy (method of making) and a chimeric antibody. This group comprises a product and 2 methods to comply with the rules under PCT and does not require the use of any of the other groups listed. Additionally, applicant discloses on page 4, lines 16-21) that "the work of the present inventors leading to the present invention is briefly described in an abstract by Tsang et al., "Monoclonal Antibodies to Human Colon Carcinoma Associated Antigens," Intl. Symp. Biotech. in Clin. Med., Rome, Italy, April 13-15, 1987. This reference was made available to the public less than one year before the filing of the ultimate parent application (U. S. Serial No. 07/176,337) for the present application. Application SN 07/176,337 was filed 31 March 1988 and the instant PCT application has priority back to 12 September 1994, therefore, the special technical feature of the instant application is already known in the art and the further payment of additional fees for searching the additional inventions is considered to be proper. Group II is drawn to an antigen which is broadly claimed as no sequence information is available, allowing for a multitude of epitopes to be available for producing antibodies other than those of Group I. Group III is drawn to an imaging method which uses a broadly claimed antibody of claim 1 which is additionally radiolabelled, thus could include an antibody to any epitope of the colon carcinoma-associated protein antigen. Group IV is drawn to a method of killing cells and a method of treating using an effective amount of an antibody. This method fails to specifically require the use of a single monoclonal antibody and could be satisfied with an antibody meeting the breadth of claim 1. Group V is drawn to a vaccine comprising a human colon carcinoma associated protein antigen as broadly described in claim 15 and is not limited to a specific sequence. Group VI is drawn to a method of immunotherapy by administering a vaccine comprising a human colon carcinoma associated protein antigen as broadly defined in claim 15. The method of treating does not require a specific antigen as defined by an amino acid sequence. Therefore, Groups I-VI are independent groups and do not require any other the other groups.